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# Cellular and molecular cross talk between basophils and CD4+ T cells

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Meenu Sharma. Cellular and molecular cross talk between basophils and CD4+ T cells. Biotechnology. Université de Technologie de Compiègne, 2014. English. NNT : 2014COMP1771 . tel-01066885

**HAL Id: tel-01066885**

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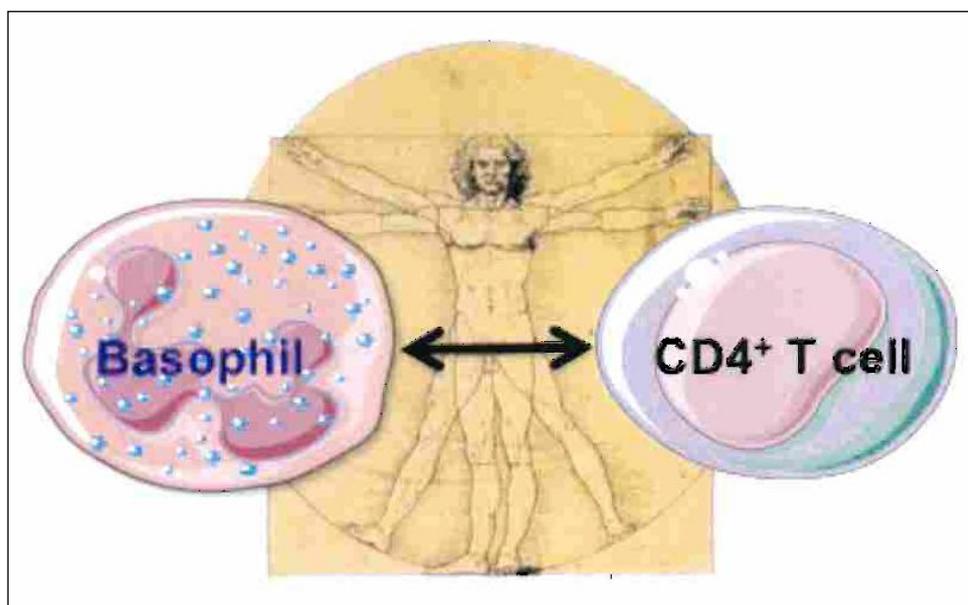
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Par **Meenu SHARMA**

*Interactions cellulaires et moléculaires entre  
basophiles et lymphocytes T CD4+*

Thèse présentée  
pour l'obtention du grade  
de Docteur de l'UTC



Soutenue le 26 mai 2014  
**Spécialité** : Biotechnologie

D1771

# Université de Technologie de Compiègne



**Champ disciplinaire: Biotechnologie**

Thèse présentée par

**Meenu SHARMA**

Pour l'obtention du grade de Docteur de l'UTC

Sujet de la thèse

**Interactions cellulaires et moléculaires entre basophiles et lymphocytes T CD4<sup>+</sup>**

**Thèse dirigée par : Dr. Jagadeesh BAYRY**

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**Soutenue le : le 26 Mai 2014**

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## Acknowledgements

I wish to take this opportunity to express my sincere gratitude to **Srini Kaveri** for giving me the opportunity to pursue my PhD in Equipe 16. He has been a great guiding force and a motivator for me during the entire period of my PhD. The moral boost-up sessions with him always encouraged me to do better each time. His consistent support and care during difficult times, is really admirable. Apart from science, his sincere efforts to make our stay comfortable as well as memorable is remarkable. I will treasure life long the enthusiastic discussions with him about music, cinema and philosophy.

I would like to express my deepest appreciation to my thesis supervisor **Jagadeesh Bayry**, for his kind support, consistent guidance and inspiration to bring perfection in all aspects of my work. His precious suggestions and criticism has contributed a lot in the overall development of my scientific and professional skills. I thank him for being patient and supportive through my entire stay. I admire the way he understands the value of hard work and always make sure about encouragement of the students. The philosophy of him that in science nothing is positive or negative and that all scientific observations are equally important, has changed my perception and gave me the strength to always pursue further for something new. I believe all that I have learnt from him will surely help me in my career.

My special thanks to **Dr. Alain Friboulet** for being a wonderful co-supervisor. His kind, prompt and humble support has been very crucial all these years. I would like to convey my sincere gratitude to **Dr. Bérangère Bihan-Avalle** for agreeing to be the president of the jury. My sincere thanks to **Dr. Kithiganahalli Narayanaswamy Balaji** and **Dr. Hélène Dumortier** for agreeing to be the rapporteurs of my thesis. I would also like to convey my kind regards to **Dr. Pascal Poncet** for agreeing to be the examinateur of my thesis.

I wish to express sincere thanks to **Sébastien Lacroix-Desmazes**, for his precious advises. My work has greatly benefited by his critical suggestions during lab presentations. The assistance and co-operation of **Mme Marie-Francoise Bloch** and **Mme Véronique Barraud** and **Mme Sylvie Carlier (UTC)** in making all the official work easier is immense. I wish to express my deepest gratitude for their consistent efforts, care and support.

I had great pleasure working with my labmates and friends, without whom this journey would have been impossible. Since, I came from a different scientific background, I owe all my technical expertise to **Pushpa** and **Mohan** for training me so patiently and guiding me unconditionally. I wish to convey my thanks to **Chaitrali** and **Emmanuel** for their consistent aid all the time. Working with them has always been a great fun and I will cherish lifelong the moments all three of us have shared together. I wish to thank **Sahana** for the short but wonderful time we spent together. My special thank to **Julie and Maxime** for their unconditional and kind help at various occasions. I wish to also thank **Sandrine, Selma, Shiva, Jordan, Mathieu, Nimesh, Ivan, Cyril, Sebastien (iSeb), Maud, Bagi, Laurent and Justa**. Your support has been invaluable all these years.

I would like to thank my soul-mate Ankit for always being there to give the strength to my weaknesses. I also wish to convey my deepest gratitude, love and respect to my parents, my family and family-in-law. I also thank my brother Gaurav and sister Richa for consistently being great source of strength and positivity. The little I have achieved is because of blessings of loved ones. I owe all my success to them; without their love and selfless efforts this would not have been possible.

At last, I thank Indo-French Centre for the Promotion of Advanced Research (CEFIPRA) and European Community's Seventh Framework Programme ( [FP7/2007-2013] under Grant Agreement No: 260338 ALLFUN) for providing doctoral fellowship .

## **Résumé en français**

**Interactions cellulaires et moléculaires entre basophiles et lymphocytes T CD4+**  
par

**Meenu SHARMA**

<< Biotechnologie et mise en œuvre des Fonctions Biologiques >>

Thesis is presented at the Faculty of the Université de Technologie de Compiègne for obtaining the degree of Doctor of Philosophy (Ph.D) of the Université de Technologie de Compiègne

**Les mot-clés en Français** – Basophiles, lymphocyte T, maladies auto-immunes, maladies inflammatoires.

Les basophiles sont les granulocytes les plus rare. Ils sont impliqués dans la polarisation des réponses immunitaires de type Th2, dans la différenciation des lymphocytes B et dans la protection contre les infections helminthiques. Les basophiles sont impliqués dans la modulation des réponses immunitaires, en particulier dans les maladies auto-immunes et inflammatoires. Des études récentes ont montré que les basophiles murins sont cellules présentatrices d'antigène (CPA) et induisent des réponses Th2 et IgE contre les allergènes et les infections helminthiques. Par conséquent, Nous avons exploré les fonctions des basophiles humains, en particulier comme CPA professionnelles. Les résultats montrent que les basophiles, contrairement aux cellules dendritiques et monocytes, n'expriment pas HLA-DR et les marqueurs de co-stimulations CD80 et CD86. De plus, la stimulation des basophiles par divers allergènes, comme des ligands de TLR et IgE, n'induit pas des changements dans l'expression de ces marqueurs. Enfin, nos résultats montrent que les basophiles ne favorise pas les réponses immunitaire de type Th2 ou Th17. Ainsi, notre étude montre que les basophiles humains circulant ne possèdent pas des fonctions de CPA professionnelles.

Des plus, les basophiles sont impliqués dans la pathogenèse de maladies auto-immunes et inflammatoires dépendantes des réponses Th2 et médiées par les lymphocytes B. Puisque la dérégulation des basophiles joue un rôle important dans le développement des réponses immunitaires dans différentes conditions pathologiques, nous avons exploré les mécanismes de régulations qui modulent les fonctions des basophiles. En particulier, nous avons étudié le rôle suppresseur des lymphocytes T régulateurs (Tregs) CD4+CD25+FoxP3, des cellules clés dans la maintenance de l'homéostasie immune, sur les fonctions des basophiles. Nos résultats montrent que les fonctions des basophiles, contrairement à la majorité des cellules immunes, ne sont pas régulées par les Tregs. Bien au contraire, nos résultats montrent que les lymphocytes T favorisent l'activation des basophiles.

En résumé, nous avons exploré de nouveaux mécanismes cellulaires et moléculaires impliqués dans la régulation des fonctions des basophiles humains. Ces résultats nous permettent de mieux comprendre le rôle des basophiles dans les conditions inflammatoires et dans le développement de nouvelles stratégies thérapeutiques.

## **Résumé en Anglais**

### **Cellular and molecular cross talk between basophils and CD4+ T cells**

by

**Meenu SHARMA**

<< Biotechnologie et mise en œuvre des Fonctions Biologiques >>

This thesis is presented at the Faculty of the Université de Technologie de Compiègne for obtaining the degree of Doctor of Philosophy (Ph.D) of the Université de Technologie de Compiègne

**Les mot clés en Anglais** – Basophils, T lymphocytes, auto-immune diseases, inflammatory diseases.

Basophils are the rare granulocytes and play an important role in the polarization of Th2 responses, differentiation of B cells and protection against helminths. Basophils have a major influence on immune responses and various roles of these cells in autoimmune and inflammatory diseases are emerging. Recent reports showed that murine basophils function as antigen presenting cells (APCs) to induce Th2 and IgE responses to allergens and helminths. Therefore, I explored whether human basophils possess the features of APCs. I found that unlike dendritic cells (DCs) and monocytes, steady-state circulating human basophils did not express HLA-DR and co-stimulatory molecules CD80 and CD86. Basophils remained negative for these molecules following stimulation with various allergens, toll-like receptor ligands and IgE cross-linking. Unlike DCs, basophils did not promote Th2 and Th17 responses. Together, these results demonstrate the inability of circulating human basophils to function as professional APC. Further, basophils were also reported to be implicated in the pathogenesis of Th2 –associated and B cell-mediated autoimmune and inflammatory diseases. Considering the impact of dysregulated function of basophils on the outcome immune responses in various pathological conditions, it was essential to investigate the regulatory mechanisms by which basophil functions are kept in check.

As  $CD4^+CD25^+FoxP3^+$  regulatory T cells (Tregs) are critical for the maintenance of immune homeostasis, I sought to investigate the interaction of Tregs with human basophils and its repercussion on basophil functions. My results indicated that unlike other immune cells that are susceptible to Treg-mediated suppression, basophils are refractory to regulatory mechanism of Tregs. On the contrary I found that T cells could promote activation of basophils.

My results thus provided an insight on cellular and molecular basis of regulation of human basophil functions. These data will have a repercussion in understanding role of basophils in inflammatory conditions and in designing therapeutic strategies.

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# Abbreviations

**APC:** Antigen presenting cells

**BAFF:** B cell activation factor

**BCR:** B-cell receptor

**BMCP:** Basophil-mast cell precursor

**C5a:** Complement component 5a

**CD:** Cluster of differentiation

**CMP:** Common myeloid progenitor

**CTL:** Cytotoxic T lymphocytes

**DC:** Dendritic cells

**EBP $\alpha$ :** Enhancer-binding protein-alpha

**EoE:** Eosinophilic esophagitis

**GALT:** Gut-associated lymphoid tissue

**GM-CSF:** Granulocyte macrophage colony-stimulating factor

**GMP:** Granulocyte-monocyte progenitor

**H<sub>2</sub>O<sub>2</sub>:** Hydrogen peroxide

**HDM:** House dust mite

**HIDS:** Hyper-IgD syndrome

**HLA:** Human leukocyte antigen

**HSC:** Hematopoietic stem cell

**IFN:** Interferon

**IgD:** Immunoglobulin D

**IgE:** Immunoglobulin E

**IL:** Interleukin

**IRD:** IgG4- related diseases

**LT-HSC:** Long term reconstituting hematopoietic stem cell

**MALT:** Mucosal-associated lymphoid tissues

**MEP:** Megakaryocyte-erythrocyte progenitor

**MHC:** Major histocompatibility complex

**MWS:** Muckle-Wells syndrome

**NK:** Natural killer cell

**NKT:** Natural killer T cell

**NO:** Nitric oxide

**O<sub>2</sub><sup>-</sup>:** Superoxide anion

**PAF:** Platelet activation factor

**PAMPs:** Pathogen-associated molecular patterns

**PAR:** Protease-activating receptor

**PFAPA:** Periodic fever-aphthous stomatitis-pharyngitis-cervical adenitis

**PGN:** Peptidoglycan

**PRRs:** Pattern recognition receptors

**SLE:** Systemic lupus erythematosus

**ST-HSC:** Short-term reconstituting hematopoietic stem cell

**TCR:** T-cell receptor

**TGF:** Tumor growth factor

**Th:** T-helper cells

**TLR:** Toll-like receptor

**TNF:** Tumor necrosis factor

**TRAPS:** TNF receptor-associated periodic fever syndrome

**Tregs:** Regulatory T cells

**TSLP:** Thymic stromal lymphopoietin

**VCAM:** Vascular cell adhesion molecule

## **L' intitulé de l'unité**

**Immunopathologie et Immunointervention Thérapeutique**

## **L'adresse de l'unité où la thèse a été préparée**

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# Introduction

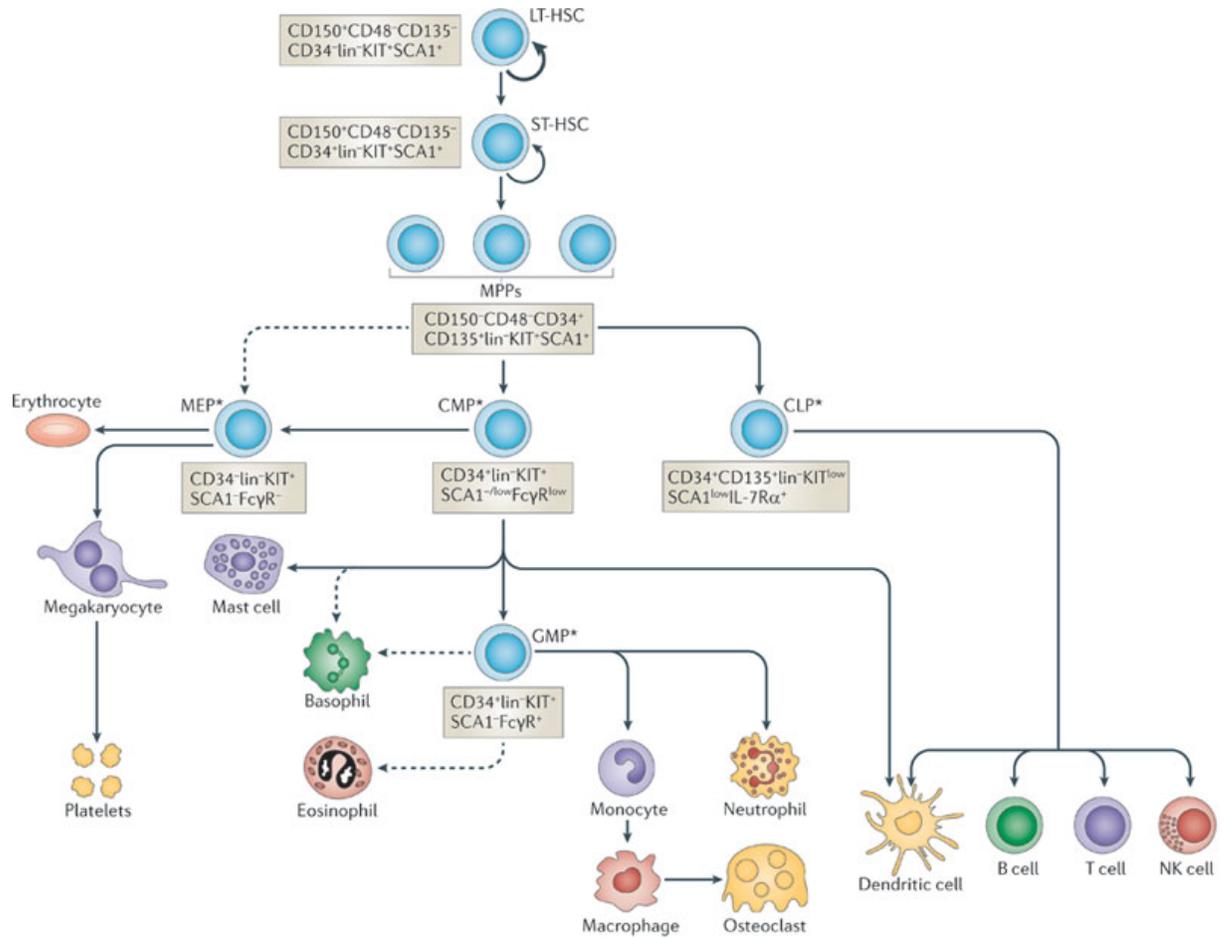
## 1. Immune system and Immune response

Immune system is a defense mechanism to protect the host from the invasion of pathogenic organisms. It is a dynamic network of cellular and molecular components that can recognize self versus non-self elements and mount effector response to eliminate the foreign components. Thus, an immune response is an array of defense mechanisms involving cells and chemical mediators of immune system that act together to provide protection against pathogen without being harmful to the host. Immune response can be functionally separated into two related activities—recognition and response. **Immune recognition** is remarkable for its specificity by which immune system recognizes subtle chemical differences that distinguish one foreign pathogen from another. Upon recognition, it mounts an appropriate **effector response** to eliminate the foreign invaders.

Immune system possesses *diversity*; to respond against enormous variety of pathogens as well as *specificity*; to mediate appropriate and efficient responses against the particular pathogen. These unique functions of immune system are executed by different cellular and molecular components, which can be classified into ‘innate’ and ‘adaptive’ immune compartments (Kuby 2006). Although, immune system employs highly regulated defense mechanisms but in certain conditions, the dysregulation either in the self- non self recognition or an excessive immune response might lead to autoimmune and inflammatory diseases and could become detrimental to host itself.

## 2. Ontogeny and hematopoiesis of immune cells

The cells of immune system are derived from a single category of cells known as pluripotent hematopoietic stem cells (HSC). The hematopoietic stem cells are able to differentiate and give rise to different lineages that generate, erythrocytes, granulocytes, monocytes, dendritic cells (DCs), macrophages, natural killer (NK) cells, mast cells, lymphocytes and megakaryocytes. The process by which formation of blood cellular components occur is called as hematopoiesis. It begins in the initial weeks of embryo development in the embryonic yolk. The hematopoietic stem cells migrate from the yolk sac to the fetal liver and then to the spleen in the initial phase of gestation. In the later phases, bone marrow becomes the major organ for hematopoiesis. A pluripotent stem cell differentiation leads to either a **common lymphoid progenitor cell** or a **common myeloid progenitor cell**. The types and amounts of growth factors in the microenvironment govern the further differentiation of progenitor cells. Common lymphoid progenitor cells give rise to B cells, T cells, and NK cells. Whereas, myeloid stem cells generate progenitors of red blood cells, and white blood cells such as neutrophils, eosinophils, basophils, monocytes, macrophages, DCs, mast cells and platelets (Figure 1). White blood cells pass through bone marrow and enter the circulation where they encounter antigens. After hematopoiesis, maturation of the lymphocytes takes place in **primary lymphoid organs**; thymus and bone marrow. The development and maturation of T-cell take place in the thymus, while B cells develop in the bone marrow. The site where these mature lymphocytes could interact with antigens are collectively called **secondary lymphoid organs**, that include lymph nodes, spleen, and various mucosal-associated such as gut-associated lymphoid tissue (GALT).



**Figure 1:** Schematic representation of process of hematopoiesis, describing lineage of different immune cells. Hematopoietic stem cells (HSCs) are divided into at least two subsets: long-term reconstituting HSCs (LT-HSCs) and short-term reconstituting HSCs (ST-HSCs). They exhibit more-limited self-renewal potential. Further differentiation of ST-HSCs generates multipotent progenitors (MPPs) and then oligopotent progenitors that include common lymphoid progenitor (CLP), common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-monocyte progenitor (GMP) populations. These populations then lead to generation of different immune cells. (Adapted from: Wang et al., 2011 Nature reviews molecular cell biology) (Wang and Wagers, 2011)

### **3. Innate and adaptive immunity**

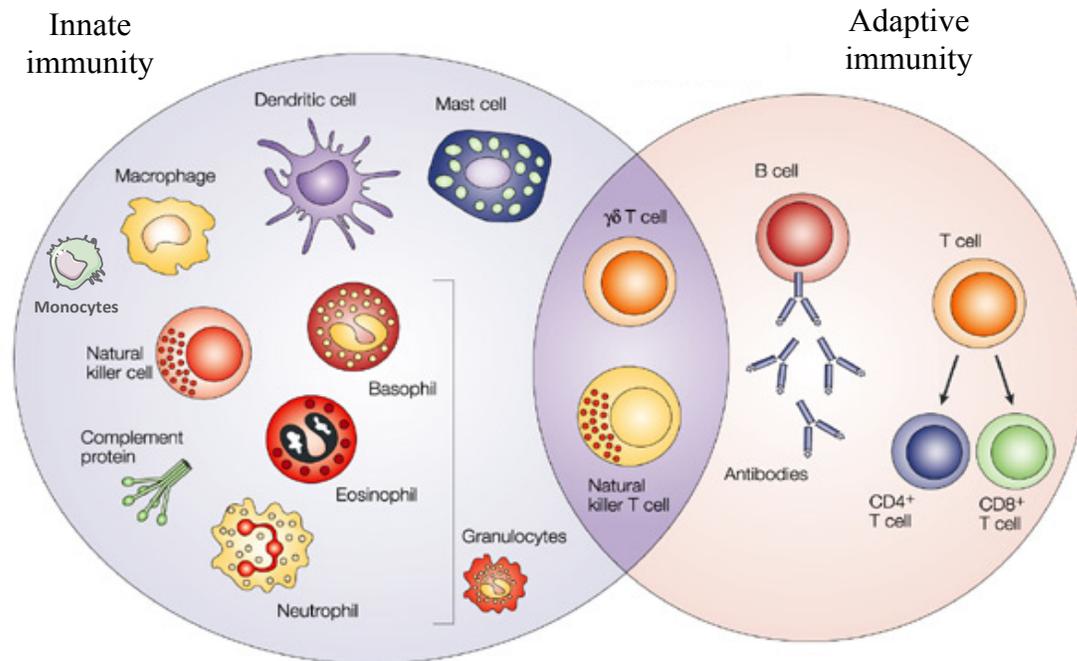
**Innate immunity** provides the first line of defense and has a strong cellular network consisting of polymorphonuclear cells like neutrophils, eosinophils and basophils as well as mononuclear cells such as monocytes, mast cells, DCs, macrophages and NK cells (Figure 2). Innate immune cells are the first to sense danger signals from invading pathogens and mount a response to eradicate them. Responses from the innate immune system are triggered upon pathogen recognition by a set of pattern recognition receptors (PRRs) expressed on the surface and cytoplasm of innate immune cells. The pathogen-derived antigens recognized by these PRRs are collectively known as pathogen-associated molecular patterns (PAMPs) which represent molecular structures that are expressed only by the microbial pathogens, but not by the host (Medzhitov and Janeway, 2000b).

Macrophages are the first to encounter pathogens in the tissues but they are soon reinforced by the recruitment of large numbers of neutrophils to sites of infection. Upon pathogen recognition through PRRs, antigen is phagocytized by an active process of internalization in a membrane-bound vesicle known as a phagosome. Phagocytic cells such as macrophages and neutrophils have lysosomes that contain enzymes, proteins, and peptides, which can mediate intracellular antimicrobial responses. Upon phagocytosis, macrophages and neutrophils also produce a variety of other toxic products such as hydrogen peroxide ( $H_2O_2$ ), the superoxide anion ( $O_2^-$ ), and nitric oxide (NO), which facilitate killing of the pathogens.

Macrophages activated by the exposure of microbial molecules such as endotoxin, also produce pro-inflammatory cytokines, including IL-6, TNF- $\alpha$ , and chemokines. Further, the granulocytes are characterized by their distinctive cytoplasmic granules and classified as neutrophils, eosinophils and basophils. Eosinophils are phagocytic and motile cells that can migrate from the blood to the tissue spaces and could secrete contents of granules containing reactive oxygen species that facilitate damage of the parasite membrane. Basophils are non-phagocytic granulocytes that function by releasing pharmacologically active substances from their cytoplasmic granules as well as variety of cytokines that could regulate adaptive immune responses. Eosinophils and basophils, both play crucial role in providing protective immunity against parasitic infections. Further, mast cells could be found in a wide variety of tissues, including the skin, connective tissues of various organs, and mucosal epithelial tissue of the respiratory, genito-urinary and digestive tracts. Mast cells are often designated as tissue-resident counterpart of basophils as these cells also have large numbers of cytoplasmic granules that contain histamine and other pharmacologically active substances and play major role in allergic reactions (Medzhitov and Janeway, 2000a).

However, DCs have major function of antigen presentation to T cells and are sentinels of the immune system. DCs usually rest in the periphery in immature form wherein they express high levels of PRRs and exhibit high capacity to uptake antigens. Upon recognition and internalization, antigens are degraded into small antigenic peptides and processed in phagolysosome for presentation via MHC class II molecules. However, endogenous antigens are processed in proteosomes and presented in the context of MHC class I. Therefore, ligation of PAMPs through PRRs could trigger the maturation and

activation DCs. Mature DCs express high levels of antigen–MHC complexes, co-stimulatory molecules and produce variety of cytokines that facilitate the differentiation of CD4<sup>+</sup> T helper (Th) cells.



**Figure 2:** Cellular components of innate and adaptive immune system

(Adapted from: Dranoff , 2004 Nature Reviews Cancer ) (Dranoff, 2004)

**Adaptive immune system** responds to the antigen challenge with a high degree of specificity. Adaptive immune system also exhibits the remarkable property of memory. A sequential acquisition of immunologic memory is achieved after—initial antigen recognition that enables cells of the adaptive compartment to pose a heightened, rapid and more efficient immune response upon re-exposure to the same antigen (Clark and Kupper, 2005).

Adaptive immune response essentially carried out by two major populations of lymphocytes; T cells and B cells (**Figure 2**). T cells develop in thymus and are primarily responsible for cell-mediated immune responses. B cells that develop in bone marrow are involved in both cell-mediated and humoral immune responses. T cells are further categorized into CD4<sup>+</sup> T helper cells (Th), and CD8<sup>+</sup> cytotoxic T lymphocytes (CTL). In addition, there is a minor population of  $\gamma\delta$  T cells and NKT cells which share properties of T cells and NK cells (Chien et al., 1996).

The CD4<sup>+</sup> Th cell responses are carefully regulated as it can only recognize antigen that is displayed along with MHC class II molecule on the surface of APC. Thus, recognition and internalization of antigens by APCs are the primary events in the initiation of immune response. Macrophages, DCs and B cells have been identified as professional APCs and could encounter the antigen in the peripheral tissues, circulation or in lymphoid organs.

DCs undergo maturation upon antigen internalization and migrate to secondary lymphoid tissues where they present antigenic peptides to naïve CD4<sup>+</sup> T cells (in the context of MHC class II) or CD8<sup>+</sup> cytotoxic T cells (CTLs; in the context of MHC class I). The T-cell activation occurs through various signals received via APC interaction involving,

interaction of antigen-MHC-II complex on APC with T-cell receptor TCR and (signal 1), binding of accessory co-stimulatory molecules such as CD80/CD86 and CD40 expressed by APCs to CD28 and CD40L (CD154) on T cells (signal 2). The co-stimulatory interactions provide further strength to the cognate interaction of APC and T cells. An additional signal in the form of immune-modulatory cytokines (signal 3) produced by activated APC direct the polarization of CD4<sup>+</sup> T cells towards Th1, Th2, Th17 or induced CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (iTregs) (Aimanianda et al., 2009).

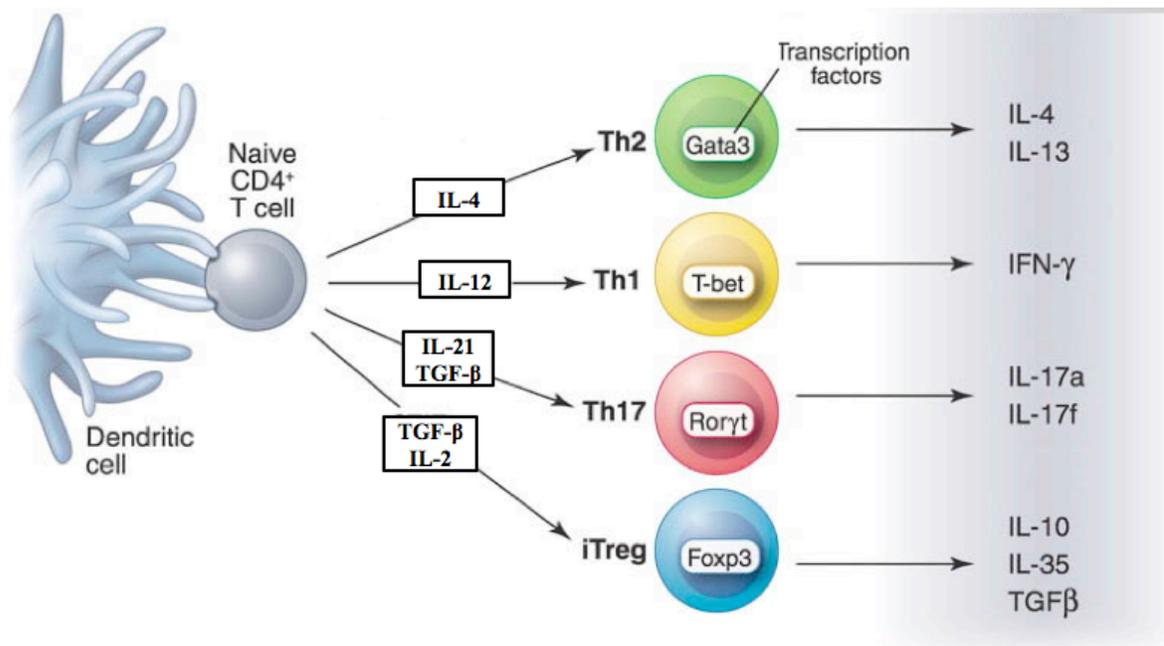
In this context, interleukin (IL)-12 has an important role in the differentiation of Th1 cells, whereas IL-4 is crucial for Th2 differentiation. IL-21 along with transforming growth factor (TGF)- $\beta$  promotes the differentiation of human Th17 cells from naïve CD4<sup>+</sup> T cells. The Treg population includes cells of thymic origin that could be expanded in the periphery upon recognition of antigens and these cells are designated as ‘natural’ Tregs (Sakaguchi et al., 2006). However, IL-2 and TGF- $\beta$  are crucial for differentiation from naïve T cells to a population of Tregs, called ‘induced’ or ‘adaptive’ Tregs (Chen et al., 2003a) (Figure 3).

Each of these CD4<sup>+</sup> T cell subsets is characterized by their specific transcription factors and production of signature cytokines (Zhou et al., 2009). Th1 differentiation is directed under transcriptional control of T-bet (**Figure 3**). Th1 cells produce interferon- $\gamma$  and are crucial for protection against several viruses and intracellular pathogens. Th2 cells are marked by expression of transcription factor GATA3 and the production of IL-4, IL-5 and IL-13 (**Figure 3**). Th2 cells are critical for clearing extracellular pathogens and parasites and are usually associated with humoral or antibody mediated reactions. Further, Th-17 cells are governed by transcriptional control of RORC in human and

ROR $\gamma$ t in mice. These cells produce IL-17A, IL-17F, IL-21 and IL-22 and are crucial for protection against several extracellular bacteria and fungi (Dong, 2008). However, Tregs express transcription factor Foxp3 and could secrete IL-10 and TGF- $\beta$ . Tregs play an essential role in maintaining self-tolerance and in regulation of immune homeostasis. Tregs regulate the function of various immune cells by exerting suppressive functions through contact-dependent as well as  $\beta$ -independent mechanisms.

Another crucial component of the adaptive immune system are B cells. Mature B cells express a membrane-bound antigen-specific antibody molecule that serves as a B-cell receptor (BCR). Mature naïve B cells undergo activation upon recognition of an antigen. However, antibody responses to antigens require co-operation between the antigen-specific B cells and helper T cells (McHeyzer-Williams and McHeyzer-Williams, 2005). The interaction between T cells and B cells sequentially involves antigen internalization by B cells and then presentation of the processed antigen to Th cells. Further, a co-stimulatory signal is provided by membrane molecules on activated Th cells such as CD40L that bind to CD40 expressed by B cells. Once activated, the B cell begins to express membrane receptors for various cytokines, such as IL-4, IL-5 and IL-13 produced by the helper T cells. Therefore, under the influence of CD4<sup>+</sup> Th cells, these antigen-specific B cells undergo isotype class switching and affinity maturation, which enhances the specificity of antibody towards an antigen leading to the generation of memory B cells and antibody-producing plasma cells.

In summary, activation of adaptive immune components depends on a relay of signal from innate cells. Upon activation, adaptive immune cells could then regulate the further response from innate immune cells. Thus, both innate and adaptive immune responses are highly coordinated and intensely regulated that complement each other.



**Figure 3:** Differentiation of CD4<sup>+</sup> T cells. Following interaction with antigen presenting cells (APC) such as dendritic cells (DC), macrophages and B cells, the activated naïve T cells can differentiate into distinct T cell subsets based on the cytokine milieu in the local environment.

(Adapted from: O'Shea and Paul, 2010 Science) (O'Shea and Paul, 2010)

## 4. Basophils as innate immune cells

Basophils are rare granulocytes and represent less than 1% of total circulating leukocytes. Basophils are identified by expression of surface markers including FcεRI, CD203c, CD49b (DX5), CD123 (IL-3 receptor α chain) and CD200R3 (a disulfide-linked dimeric CD200R-like receptor belonging to the immunoglobulin superfamily) and c-Kit<sup>-</sup> (CD117).

### 4.1 Development of basophils

Basophils discovered by Paul Ehrlich in 1879, based on their unique microscopic appearance upon staining with basic dye (Ehrlich, 1879). Basophils arise from common myeloid progenitor and develop from granulocyte-macrophage progenitor (GMP) cells. Their further lineage commitment is strictly regulated by the timed expression of transcription factors GATA-2 and CCAAT/enhancer-binding protein-α (C/EBPα) (Iwasaki et al., 2006) (Voehringer, 2013). Down-regulation of GATA-2, accompanied by enhanced expression of C/EBPα in GMP leads to generation of bi-potent basophil-mast cell precursor (BMCP) as demonstrated in mice. However, *in vitro* experiments suggested that complete deletion of C/EBPα in purified BMCP, promoted mast cell development, whereas overexpression of C/EBPα supported the development of basophil lineage (Arinobu et al., 2005). Further, STAT5 (signal transducer and activator of transcription 5)-mediated signals were reported to be critical for the development of LIN<sup>-</sup> CD34<sup>+</sup> KIT<sup>-</sup> FcεRIα<sup>+</sup> β7integrin<sup>low</sup> basophil precursors in the bone marrow and BMCPs in the spleen (Ohmori et al., 2009). In a steady state condition, basophil production primarily occurs in the bone marrow, however they can also develop from

progenitors in extra-medullary tissues under inflammatory diseases (Voehringer, 2013) (Ohnmacht et al., 2010). Basophils enter the peripheral blood upon completion of maturation in the bone marrow and do not proliferate further.

Cytokines and other factors have been reported to play critical role in basophil differentiation and development. IL-3 and TSLP (thymic stromal lymphopoietin) are the two cytokines that were shown to promote basophilia by activating STAT 5 (Didichenko et al., 2008) (Perrigoue et al., 2009). STAT5-mediated induction of the serine/threonine kinase PIM1 was shown to play a role in rescuing basophils from undergoing apoptosis (Didichenko et al., 2008) (Perrigoue et al., 2009). Further, *in vitro* treatment of bone marrow precursors of basophils in mice with IL-3 was shown to increase *de novo* production of basophils indicating direct effect of IL-3 on basophillia (Siracusa et al., 2011) (Ohmori et al., 2009). However, in a separate study, IL-3 was found to be crucial for parasite-induced basophil production and not for the survival *in vivo* (Shen et al., 2008). Therefore, IL-3 reported to rapidly promote basophillia by inducing *de novo* basophil production as well as by inhibiting apoptosis of mature basophils, though effects of IL-3 on basophil survival *in vivo* during infection require further exploration.

Treatment of bone marrow-derived basophil precursors with TSLP and IL-3, showed increase in basophil number upon treatment with IL-3 but not with TSLP treatment. However, basophil survival was enhanced by TSLP (Siracusa et al., 2011). This report suggested that TSLP might contribute in enhancement of basophil number mostly by promoting survival of mature basophils instead of inducing production. As IL-3, GM-CSF (granulocyte–macrophage colony-stimulating factor) and IL-5 receptors are known to have shared  $\beta_c$  chain, it was reported that GM-CSF and not IL-5 could also induce

basophil expansion in mice, in a low IL-3 environment (Schneider et al., 2009). Apart from this, IL-33 was also identified to indirectly promote basophil development by inducing GM-CSF and IL-3 in bone marrow cells, upon *in vivo* administration (Schneider et al., 2009). In conclusion, IL-3 and TSLP have been identified as major cytokines directing development of basophils from precursor cells.

## **4.2 Mechanisms of basophil activation**

Basophils can readily be activated by an array of signals including cytokines, antibodies and proteases (Siracusa et al., 2013). Basophil activation is marked by enhanced expression of activation markers like CD203c, CD63 and CD69 as well as secretion of vasoactive mediators such as histamine and leukotrienes, and cytokines including IL-4, IL-13, IL-6 and TSLP.

### ***4.2.1 Cytokine-mediated basophil responses***

So far, IL-3 has been identified as major cytokine having potent impact on basophils development, survival, migration as well as priming for efficient IgE-mediated stimulation. It was reported that IL-3 priming could significantly enhance IL-4, IL-13 and histamine release from basophils followed by anti-IgE stimulation in human and direct IL-4 release in mice (Silver et al., 2010) (Qi et al., 2010) (Schroeder et al., 2009) (Brunner et al., 1993) (Schroeder et al., 1994). In addition, IL-33 and IL-18 were also been shown to exert stimulatory effect on basophils promoting release of Th2-associated cytokines IL-4, IL-13 as well as histamine release in My-D88 dependent manner (Kroeger et al., 2009). However, human basophils did not respond to IL-18 treatment

thus suggesting differences among human and murine basophils in their ability to respond to cytokines (Yoshimoto et al., 1999) (Yoshimoto and Nakanishi, 2006). Furthermore, IL-3 treatment was shown to enhance the expression of ST2, the activating receptor subunit for IL-33 on basophils (Pecaric-Petkovic et al., 2009).

IL-33 was reported to synergize with IL-3 for basophil activation and for the release of soluble mediators. However, IL-33 and IL-3 utilize distinct signaling pathways to mediate activation of basophils. IL-3 activates JAK/STAT pathway and ERK activation in human basophils whereas; IL-33 activates NF $\kappa$ B and p38 MAP-kinase pathway (Pecaric-Petkovic et al., 2009). Also, unlike IL-3, IL-33 could not prime human blood basophils for C5a-induced leukotriene C4 generation (Pecaric-Petkovic et al., 2009).

*In vivo* studies in mice demonstrated that TSLP could also play a critical role in basophil responsiveness. Although, in a murine model of *Trichuris muris* infection, both IL-3 and TSLP seem to function in a cooperative manner to induce optimum basophil response, TSLP however showed the capability to promote basophil development and peripheral basophilic in an IL-3 independent manner (Siracusa et al., 2011). It was found that IL-3-elicited basophils are distinct from TSLP-elicited basophils in terms of their responsiveness towards cytokine stimulations as well as their migratory properties. IL-3-elicited basophils degranulated in response to IgE-mediated cross-linking but could not respond efficiently to IL-3, IL-18, or IL-33 stimulation. However, TSLP-elicited basophils possessed limited capability to degranulate upon IgE-mediated stimulation, though were capable of responding robustly to IL-3, IL-18, or IL-33 stimulation (Siracusa et al., 2011) (Siracusa et al., 2013).

#### ***4.2.2 Antibody-mediated basophil responses***

Basophils express receptors of antibodies, IgG and IgE. Activating IgG receptors are, Fc $\gamma$ RIIIA in mice and Fc $\gamma$ RIIA in humans (Cassard et al., 2012). Whereas, inhibitory receptors Fc $\gamma$ RIIB expressed in both humans and mice (Meknache et al., 2009). Binding to IgG or immune complexes to Fc $\gamma$ RIIIA, reported to trigger degranulation of basophils to a similar extent as induced by ligation of IgE receptor Fc $\epsilon$ RI, in mice (Cassard et al., 2012). However, in human Fc $\gamma$ RIIA has been found to trigger weak responses, of a much lower magnitude than induce by Fc $\epsilon$ RI, even after IL-3 priming. Further, human basophils express inhibitory Fc $\gamma$ RIIB to a higher extent than other blood leukocytes, including B cells. Recently, Fc $\gamma$ RIIB was also found to be expressed on human basophils (Meknache et al., 2009; Cassard et al., 2012). The proportion of Fc $\gamma$ RIIB+ basophils in normal donors ranges from 25-100% and reported to be decreased significantly in patients with atopic dermatitis (Meknache et al., 2009). Further, Cassard et al., recently reported that co-engagement of both inhibitory Fc $\gamma$ RIIB and activating Fc $\epsilon$ RI or Fc $\gamma$ RIIA leads to inhibition in the IgE-induced activation (Cassard et al., 2012) (Cady et al., 2010).

During first encounter of allergen or proteases, IgE antibodies generated through humoral responses circulate in peripheral blood and bind to the Fc $\epsilon$ RI on the surface of basophils. Thus, upon re-exposure, cross-linking of receptor-bound IgE by antigen (allergen) or by antigen- IgE immune complexes, trigger activation and subsequent degranulation, a process by which pre-formed pharmacologically active contents are released (Figure 4). Basophils can secrete several pre-formed mediators including histamine and proteoglycans, lipid mediators such as leukotrienes and prostaglandins and numerous

cytokines like IL-3, IL-4, IL-6, IL-9, IL-13 and IL-25 and chemokines including CCL5, CCL3, CCL4 and CCL2 (Chirumbolo, 2012).

Basophils elicit distinct responses upon activation via IgG-allergen immune complexes and mainly produce platelet activation factor (PAF) rather than histamine which lead to increased vascular permeability (Tsuji-mura et al., 2008). Therefore, authors found that basophils play crucial in the development of IgG-mediated systemic anaphylaxis in mice. In addition to IgE- and IgG-mediated activation, basophils could also undergo activation in an IgD-dependent manner. IgD-antigen complexes could induce the production of anti-microbial peptides and B cell activation factor (BAFF), APRIL from basophils (Chen et al., 2009).

#### ***4.2.3 Pattern-recognition receptor-mediated basophil responses***

Basophils express several pattern-recognition receptors (PRR) on the surface that facilitates the sensing of proteases, bacterial, viral and parasite antigens. Therefore, basophils could undergo activation by pathogen-associated molecular patterns (PAMP) in an IgE-independent manner. In this context, human basophils were reported to express high levels of PRRs of toll-like receptor (TLR) family; TLR2 and TLR4 (Sabroe et al., 2002) and low levels of TLR9 and TLR10.

Despite the presence of TLR4, human basophils did not respond to LPS-mediated stimulation (Sabroe et al., 2002) as assessed by expression of adhesion molecule CD11b, secretion of IL-8 and shedding of L-selectin. Additional parameters have also confirmed these results (Bieneman et al., 2005) wherein basophils treated with peptidoglycan, a TLR2 ligand but not LPS activated NF $\kappa$ B and induced nuclear localization of NF $\kappa$ B.

TLR-2 stimulation also induced the secretion of both IL-4 and IL-13 in an IgE-independent manner (Bieneman et al., 2005). Although peptidoglycan stimulation alone did not induce histamine or leukotriene C<sub>4</sub> release, peptidoglycan augmented the secretion of these mediators and also IL-4 and IL-13 in response to IgE-dependent activation of basophils (Bieneman et al., 2005). However, similar results were not obtained with other TLR-2 ligands such as yeast-derived zymosan and lipopeptide Pam3Cys, where only marginal increase in cytokine release from basophils was observed.

In extension to these studies, recent report showed differential response of basophils toward TLR ligands, which is based on the type of TLR that was being triggered (Suurmond et al., 2014). Additive effect on cytokine secretion was seen when basophils from allergic individuals were stimulated with TLR4 and allergen-specific IgE, indicating potential role of TLR-mediated stimulation in allergic inflammations. Supernatant from these basophils exhibited induction of Th2 polarization *in vitro* (Suurmond et al., 2014). These results suggested that human basophils could respond to TLR ligands synergistically with IgE-mediated activation, which could promote cytokine-induced Th2 differentiation.

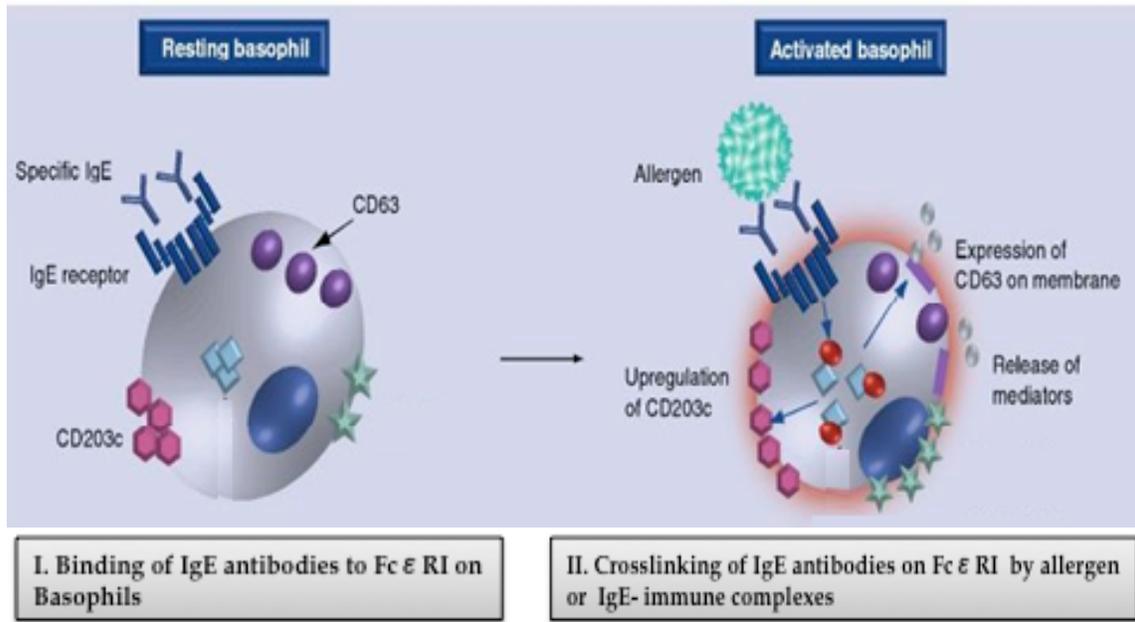
Another report demonstrated that IFN- $\gamma$  pretreatment of basophils followed by TLR4-stimulation through LPS, enhanced the expression of adhesion molecule CD11b. However, the surface expression of TLR2 and TLR4 on basophils did not change upon IFN- $\gamma$ -treatment (Komiya et al., 2006). It was found that TLR4-mediated stimulation of basophils induces B cell activating cytokines, IL-13 and BAFF and not Th2-associated IL-4 and TSLP. Further, TLR-4-mediated activation of basophils was shown to trigger IgG4

antibody production from B cells instead of IgG1 (Watanabe et al., 2013). Therefore, TLR4-induced basophil activation appears to regulate B cell functions.

However, it was reported that bone marrow-derived murine basophils selectively express TLR1, 2, 4 and 6 (Yoshimoto et al., 2009) and produce significant amounts of Th2 cytokines such as IL-4, IL-6 and IL-13, in response to IL-3 plus peptidoglycan (PGN) or IL-3 plus LPS via TLR2 or TLR4, respectively, even without FcεRI cross-linkage (Yoshimoto et al., 2009).

Further, basophils were also found to express nucleotide-binding oligomerization domain-containing protein 2 (NOD2). Ligation of NOD2 with muramyl dipeptide lead to significant increase in expression of adhesion molecules intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on basophils. This NOD2-activation was reported to facilitate basophil-bronchial epithelial cell interaction, which triggered the release of IL-6, CXCL8 and β-defensin2 from epithelial cells (Qiu et al., 2013). Thus, implied the role of NOD2-mediated basophils and epithelial cells function during innate responses against respiratory bacteria.

In addition, basophils are known to directly respond to proteases as shown by induction of IL-4 and IL-13 in basophilic cell line upon exposure of house dust mite-derived Der p 1 protease allergen (Phillips et al., 2003). Similarly, papain induced the production of Th2-associated cytokines in murine basophils (Siracusa et al., 2013) (Sokol et al., 2009). These observations thus indicated responsiveness of basophils towards active proteases. However, protease-activating receptor (PAR) expression was not detected on human basophils (Falcone et al., 2005) and hence a mechanism involved in the protease-derived human basophil responses requires further exploration.



**Figure 4:** Classical pathway of IgE-mediated basophil activation and degranulation

## 5. Basophils as antigen presenting cells

DCs were generally considered as professional APCs. However, owing to their incapability to secrete IL-4 cytokine, the role of DCs as antigen presenting cells in mediating Th2 responses was questioned (Maddur et al., 2010). Also, Th2 responses are critical for protective immune responses to multicellular helminth parasites but DCs lack the capacity to phagocytose these parasites. In a transgenic mice model of *Trichuris muris* infection, Perrigoue *et al.* observed that DC-restricted MHC-II expression was not sufficient to mount Th2 responses. As a consequence, impaired parasite clearance from intestinal tract was observed, though Th1 differentiation was found unaffected. These observations were supported by another report indicating that antigen capture by skin DCs are not required for mounting Th2 responses to the protease allergen papain, since Th2 response was observed even after removal of the antigen from the injection site (Sokol et al., 2009). To further confirm this, both the reports showed that upon selective depletion of CD11c<sup>+</sup> DCs, Th1 response was impaired, however, Th2 responses against papain or *T. muris* was not affected (Perrigoue et al., 2009). Altogether, these results indicated that DC are critical for Th1 responses but insufficient to prime Th2 responses *in vivo*, leading to exploration for the role of alternative APCs required for Th2 cytokine-dependent immunity.

In this regard, several evidences suggest the notion that basophils might act as professional APCs for mounting Th2 responses against protease, protein allergens and helminths, as basophils are known to secrete Th2-differentiating cytokines IL-4, IL-13 and TSLP and reported to express antigen presenting MHC-II molecule as well as co-stimulatory molecules CD80, CD86 that are required for priming and activation of T cells

(Perrigoue et al., 2009) (Sokol et al., 2009) (Yoshimoto et al., 2009). Further, in murine model, basophils recruitment to lymph node was observed upon papain injection as well as during infection of *T. muris*, and eggs of *Schistosoma mansoni* (Perrigoue et al., 2009), which indicated the potential of basophils to establish cognate interaction with T cells to initiate Th2 cell responses *in vivo*. These observations were substantiated by *in vitro* studies showing that basophils could promote antigen-specific CD4<sup>+</sup> T cell proliferation and Th2 differentiation in MHC class II- and IL-4-dependent manner (Perrigoue et al., 2009) (Sokol et al., 2009) (Yoshimoto et al., 2009).

Basophil depletion by monoclonal antibody to FcεRI MAR-1 showed decline in *il4* mRNA and a subsequent impairment of protective immunity against gastrointestinal helminth *T. muris* (Perrigoue et al., 2009). Further, upon adoptive transfer of papain-primed MHC class II positive basophils to MHC class II-deficient mice, antigen induced Th2 response was observed, verifying that basophils alone were sufficient to present antigens and to induce Th2 responses (Sokol et al., 2009). Though, as compared to DCs, basophils were found to endocytose only soluble and not the particulate antigens. Another study showed that basophils could also efficiently endocytose antigen-IgE complexes (Yoshimoto et al., 2009). This study reported that 2,4-dinitrophenyl-conjugated ovalbumin-immune complex (DNP-OVA-IgE-anti-DNP) could be endocytosed by basophils. Therefore, by presenting peptide MHC-II complex, basophils could induce OVA-specific Th2 responses and subsequent IgG and IgE production *in vivo* (Yoshimoto et al., 2009). Collectively, these reports suggested the role of basophils as APC in inducing Th2 responses against protease allergen, antigen-IgE complexes and helminth parasites.

However, recent study contradicted these observations by re-assessing the role of basophils as APCs in eGFP IL-4 reporter mice that were intranasally challenged with house dust mite (HDM) allergen. Lambrecht and colleagues (Hammad et al., 2010) observed that inflammatory DCs and not basophils are necessary and sufficient for induction of Th2 immunity to inhaled HDM allergen. Further, the authors demonstrated that IL-4-producing basophils were recruited to lung draining lymph nodes upon HDM inhalation, in a TLR4-MyD88 signaling-dependent manner. Interestingly, the authors observed that basophil depletion by anti-FcεRI antibodies MAR-1, strongly suppressed the levels of Th2 cytokines (Hammad et al., 2010). Whereas, depletion of basophils by using CD200R3 basophil-specific antibody Ba103 did not significantly decrease the level of Th2 cytokines. These observations suggested that the strategies to deplete basophils could greatly alter the observations regarding the role of basophils in Th2 responses. Further, the authors discovered that a distinct cell population positive for FcεRI other than basophils and mast cells, was recruited to lymph node in a greater number. This migration was observed upon challenge with HDM and not with non-specific OVA antigen. Upon flow cytometry-based phenotypic analysis, this population was identified as FcεRI<sup>+</sup>DX5<sup>-</sup> inflammatory DCs exclusively expressing CD11c and higher MHC-II as compared to basophils (Hammad et al., 2010). In a co-culture assay, sorted OVA-HDM primed FcεRI<sup>+</sup>DX5<sup>-</sup> DC induced proliferation as well as enhanced secretion of Th2 cytokines from OVA-specific CD4<sup>+</sup>T cells (Hammad et al., 2010). However, FcεRI<sup>+</sup>DX5<sup>-</sup> basophils could not take up antigen efficiently and further, did not induce proliferation and Th2 associated cytokine release from CD4<sup>+</sup> T cells. Therefore, the authors concluded that depletion by using anti-FcεRI MAR-1 antibody could non-

specifically deplete population of inflammatory DC consequently leading to low Th2 responses (Hammad et al., 2010).

In an earlier report, basophils were also shown to present peptide antigens or cross-present protein antigen to CD8 T cells, inducing IL-10-producing CD8<sup>+</sup> T cells (Kim et al., 2009). Antigen-pulsed basophils were shown to induce proliferation as well as IL-10 production from OT-I CD8<sup>+</sup> T cells, in IL-4 and IL-6 dependent manner. However, *in vivo* cytotoxic experiments failed to demonstrate regulatory properties for these IL-10 producing CD8<sup>+</sup> T cells. Also, these cells could not inhibit the proliferation of naïve CD8<sup>+</sup> T cells *in vitro* (Kim et al., 2009).

In contrast to murine reports, several studies (Kitzmüller et al., 2012) (Eckl-Dorna et al., 2012) (Poulsen et al., 2012) have strongly suggested that human basophils are incapable to act as professional APCs. Study conducted in birch pollen-allergic patients revealed that basophils from these patients are not capable to induce allergen-specific T cell responses upon secondary exposure of birch pollen allergen, Bet v 1. However, basophil depleted fraction of APCs that majorly contains CD11c<sup>+</sup> cells enhanced T cell proliferation in an *in vitro* co-culture system (Eckl-Dorna et al., 2012). Further, by using fluorescently-labeled Bet v 1, Kitzmüller et al. assessed whether basophils from birch pollen-allergic patients could bind and internalize the antigen. They found that basophils very efficiently bind to Bet v 1 through IgE/FcεRI complexes on their surface. However, basophils did not internalize allergen and marginally expressed the proteins cathepsin S and invariant chain that are crucial for antigen presentation (Kitzmüller et al., 2012). The authors could not observe expression of antigen presenting HLA-DP, HLA-DQ and Co-stimulatory molecules CD80/CD86 and CD40 on either purified basophils or IL-3 plus

IFN- $\gamma$  treated basophils. However, marginal increase in HLA-DR was observed upon cytokine treatment. Bet v 1-pulsed basophils failed to act as APCs in co-culture experiments with Bet v 1-specific T-cell clones and could not induce T cell proliferation. These results further confirmed the inability of basophils to internalize, process and present antigen in context to MHC-II to induce T cell responses in healthy as well as in IgE-mediated allergy in humans.

Apart from allergic conditions, Charles et al., demonstrated that human basophils could express MHC-II molecules and therefore are capable to act as APCs in Th2-associated autoimmune condition such as SLE (Charles et al., 2010). However, Dijkstra et al. contradicted this observation, by showing that surface markers that are frequently used to analyze basophils such as Fc $\epsilon$ RI $\alpha$  and CD123 are shared by plasmacytoid DC (pDC) and basophils. Therefore, based on phenotype CD123<sup>+</sup>CD304<sup>-</sup> as basophils and CD123<sup>+</sup>CD304<sup>+</sup> as pDC, the authors demonstrated in eight SLE patients that these are not basophils, but pDCs that identified MHC-II-expressing cells (Dijkstra et al., 2012).

A recent finding showed that up to 17% of human basophils could express MHC-II molecule upon stimulation with IL-3, IFN- $\gamma$  and GM-CSF. However, these basophils remained low positive for co-stimulatory molecules CD80 and CD86 (Voskamp et al., 2013). Despite the confirmation of up-regulation of HLA-DR, HLA-DM, CD74 and Cathepsin S in MHC Class II-positive basophils, these basophils failed to induce antigen-specific T cell activation or proliferation. Thus, further confirmed the incompetence of human basophils to act as professional APC.

## **6. Role of basophils in immune responses**

Despite being crucial component of innate immunity, basophils are often considered as a link between innate and adaptive immune response owing to their myriad functions and array of cytokines secreted by them. Basophils are predominantly found in circulation, however upon antigen challenge can also migrate to lymph node, spleen and to the site of inflammation. Therefore, basophils are capable of modulating the functions of both T and B cells either directly or indirectly.

### ***6.1 Basophils-mediated T cell responses***

Basophils can readily respond to various stimulations and produce cytokines that are critical in driving T cell responses. Basophils are initial and potent producer of IL-4 and can secrete the cytokine in response to IL-3, IL-18 and IL-33- mediated activation even without IgE-stimulation (Schroeder et al., 2009) (Voehringer, 2012) (Kroeger et al., 2009). However, along with these cytokines, IgE stimulation synergistically stimulates the IL-4 release. Therefore, basophils could contribute to skew T-cell responses towards Th2 phenotype (Figure 4). Besides these factors, epithelial cell- or mast cell-derived TSLP and various TLR2 ligands (Siracusa et al., 2011) (Sokol et al., 2009) (Bieneman et al., 2005) could also stimulate basophils to produce IL-4. Apart from IL-4, basophils are also reported to secrete TSLP, another factor that promotes Th2 polarization and thereby basophils could drive antigen-specific Th2 responses (Sokol et al. 2008). By promoting IL-4 and TSLP-mediated Th2 environment, basophils induce protective immunity against helminth infections and facilitate expulsion of parasite (Giacomin et al., 2012). Based on these reports, basophils are considered as innate regulation of Th2 responses. However,

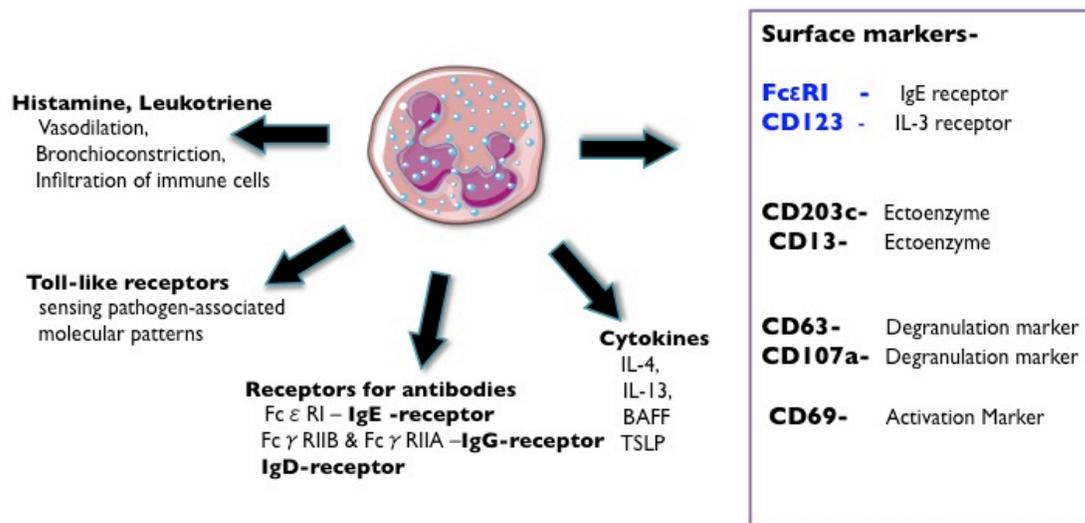
basophils were also found to suppress Th1 responses in IL-4 and IL-6 dependent manner (Gomez et al., 2014). Altogether these reports confirm the role of basophils in establishing and promoting Th2 environment. However, basophils are also recently reported to augment memory T cells response towards Th17 responses in an IL-3 and IL-33 dependent manner (Wakahara et al., 2012).

### ***6.2 Basophils-mediated B cell responses***

Recent studies have demonstrated the role of basophils in B cell activation, differentiation and antibody class switching. Thus, basophils have ability to regulate humoral responses both in human and mice (Karasuyama et al., 2011). Studies showed that basophils play a crucial role in enhancing humoral memory responses by directly providing IL-4 and IL-6 to B cells and by promoting Th2-mediated help to B cells (Figure 5) (Denzel et al., 2008). Further, in T cell-independent pathway, it was shown that TLR-activated basophils could induce IgG4 secretion from B cells both in healthy subjects and in patients with IgG4-related disease (IRD), which is characterized by infiltration of IgG4-expressing plasma cells (Watanabe et al., 2013). This B cell modulation by TLR-activated basophils was found to be mainly mediated via release of BAFF and IL-13 (Figure 5). *In vitro* studies have also indicated that human basophils could induce IgE production by B cells through expression of CD40L and production of IL-4 (Gauchat et al., 1993) (Karasuyama et al., 2011) (Yanagihara et al., 1998). As an alternative pathway, circulating IgD-antigen complexes could also stimulate basophils via cross-linking of IgD present on basophil surface (Figure 5). This axis is reported to trigger release of anti-microbial peptides, BAFF and APRIL that could activate B cells to

mount and amplify humoral response to provide protection against airway bacterial infections (Karasuyama et al., 2011) (Chen et al., 2009).

In conclusion, it is evident from several *in vitro* and *in vivo* studies that basophils upon receiving stimulatory signals from different sources such as cytokines, TLR ligands and antibodies, could mediate differential T and B cell responses and in turn are capable of regulating adaptive immune responses



**Figure 5 :** Phenotypic and functional properties of basophils

## **7. Regulation of basophil functions**

The interaction of basophils with other immune cells is not a monologue rather dialogue. While basophils regulate T cell differentiation and B cell responses, reciprocal signaling by adaptive immune compartments or from innate cells ensures optimal activation of basophils and their survival.

### ***7.1 Regulation of basophil functions by innate immune cells***

Several cytokines and other mediators secreted by innate cells are reported to regulate basophil responses. Mast cells upon activation produce IL-33, a cytokine that is reported to regulate basophil development, migration, adhesion, activation and cytokine release (Schneider et al., 2009) (Valent, 2009) (Suzukawa et al., 2008) (Silver et al., 2010) (Pecaric-Petkovic et al., 2009). Mast cells also produce IL-3 required for basophil hematopoiesis, survival and priming for IgE-mediated activation (Spiegl et al., 2008). Further, it was found that upon IgE-mediated activation, mast cells are also capable of producing TSLP, which is well-known regulator of basophil activation (Okayama et al., 2009) (Giacomin et al., 2012).

APCs such as DCs, macrophages and monocytes also exert regulatory effects on basophils. DCs were found to produce IL-33 upon activation through prostaglandin E2 (Yanagawa et al., 2011). Recent report indicated that TLR- mediated DC activation by zymosan, LPS or CpG upon ligation with TLR2, TLR4 or TLR9, respectively; could induce TSLP production (Kashyap et al., 2011) from DCs. Further house dust mite – allergen was also found to induce TSLP mRNA in murine lung DCs (Kashyap et al.,

2011). In earlier reports, CCL3 and monocyte chemoattractant protein 1 (MCP-1) were shown to induce basophil activation and histamine release (Alam et al., 1992). Further, IL-18 secreted by inflammatory monocytes and DCs in response to activation via inflammasomes was reported to induce IL-4 and histamine release from basophils (Rathinam et al., 2012) (Yoshimoto et al., 1999) (Voehringer, 2012). However IL-18 along with IL-12, another DC cytokine, reported to exhibit anti-allergic properties by inhibiting basophil responses and IgE production (Voehringer 2012; Yoshimoto et al. 1999). Though, these studies were conducted *in vivo* and showed the role of cytokines, they did not explore the consequence of direct cellular interaction between basophils and innate cells that produce IL-18 and IL-12. It was also been shown that IL-18 plus IL-12 cause enhanced release of IFN- $\gamma$  and TNF- $\alpha$  from NK cells, which promote basophils for Fas-mediated apoptosis and thereby could negatively regulate basophil response (Schneider et al., 2004) (Voehringer, 2012). In another study, IFN- $\alpha$ , type I cytokine secreted by plasmacytoid DCs have been reported to inhibit IL-3-mediated cytokine release from basophils (Farkas et al., 2001) (Chen et al., 2003b).

### ***7.2 Regulation of basophil functions by adaptive immune cells***

There are several cellular and molecular mediators of adaptive immune compartment that are known to regulate basophil development, survival and activation. Although, modulation of T cell responses by basophils has been studied vastly in past few years, the consequences of T cell effects on basophil function have not received much attention. Recently, by using IL-4 reporter mice, it was demonstrated that basophils are activated by CD4<sup>+</sup> T cell-dependent (and IgE-independent) manner and produce IL-4 in parasite-

infected tissues. In addition to direct contact with CD4<sup>+</sup> T cells, T-cell-derived IL-3 was also implicated in the activation and survival of basophils (Sullivan et al., 2011). IL-3 could directly induce low levels of IL-4 and IL-13 from human basophils, and could most efficiently promote cytokine expression in synergy with complement component 5a (C5a) or FcεRI-activation (Kurimoto et al., 1989) (Kurimoto et al., 1991). Studies have also demonstrated that IL-3 produced by activated T cells promotes basophil production *in vivo* during parasitic infection (Shen et al., 2008) (Herbst et al., 2012). However, Shen et al. also indicated the dispensability of IL-3 for basophils survival *in vivo*. Recently, TSLP-primed DCs were shown to induce IL-3 from naïve T cells via OX40L-mediated signaling. This OX40L-IL-3 axis was found to be critical for basophil recruitment and for Th2 responses (Leyva-Castillo et al., 2013).

Apart from T cells, B cells could also regulate basophil responses. Antibodies mainly mediate these functions and that different subclasses of antibodies were shown to have distinct effects on basophil functions. Upon capturing IgG-allergen complexes, basophils release platelet activation factor (PAF) leading to increased vascular permeability. However, basophils could undergo degranulation and release histamine only upon signaling by IgE-bound allergens (Karasuyama et al., 2011) (Chirumbolo, 2012). Apart from these pathways, as mentioned earlier, basophils could also respond to IgD- immune complexes that trigger the release of mediators for B cell activation (Chen et al., 2009).

## **8. Basophils in protective immune responses to pathogens**

Large multicellular organisms such as helminth parasites cannot be eliminated by phagocytosis or cytotoxic attacks (Voehringer, 2013). Helminth parasites normally

induce Th2 responses and Th2 cytokines IL-4 and IL-13 have a key role during both initiation and execution phases of the protective immunity to helminths. Basophils play a crucial role in mediating protective immune responses in these infections as basophils can be activated by proteases secreted by parasites (Phillips et al., 2003). Upon activation through either parasite-derived molecules or cytokine signals such as IL-3, IL-33, TSLP from other innate cells, basophils secrete IL-4 and IL-13 cytokines that are critical for Th2 responses and providing protection against helminth infections.

Direct protective role of basophils in helminth infection was demonstrated in a RAG2-deficient mice model of *Nippostrongylus brasiliensis* infection. In this model, selective depletion of basophils by either MAR-1 or Ba103 antibodies partially impaired the worm expulsion and was associated with a lack of cytokine production by Th2 cells (Ohnmacht et al., 2010). However, in contrast to RAG2-deficient mice, depletion of basophils in wild type mice did not diminish Th2 responses to *N. brasiliensis* that raised the questions about indispensability of basophils in protective immunity to helminth infections. These questions were subsequently answered by using Mcpt8Cre mice that are genetically deficient in basophils. By using these mice, it was demonstrated that basophils are indeed important for protection against secondary infections with *N. brasiliensis*, whereas primary Th2 cell responses could operate efficiently in the absence of basophils (Ohnmacht et al., 2010). Corroborating their protective role, similar results were obtained when basophils were depleted in mice that were infected with intestinal parasites *H. polygyrus* and *Trichuris muris* (Herbst et al., 2012) (Perrigoue et al., 2009).

Another study depicted that alternatively activated macrophages are crucial for forming granuloma around *S. mansoni* eggs and larvae to prevent excessive tissue damage.

However, differentiation of macrophages was found to be dependent on basophil-derived IL-4 and IL-13 (Herbst et al., 2012) (Anthony et al., 2006). However, in a study conducted in *S. mansoni*-infected IgE-deficient mice, smaller granuloma and higher worm burden was observed (King et al., 1997). This report thus indicated a critical role of IgE-mediated basophil activation and release of IL-4 and IL-13 in mounting protective immune response to helminth infection.

Basophils might also contribute to protective immunity to virus and bacteria by promoting B cell proliferation and antibody production. In fact, basophils have been shown to play important role in mounting humoral memory responses against *Streptococcus pneumoniae* via IL-4 and IL-6 that enhance B cell proliferation and antibody production (Denzel et al., 2008).

Activated human basophils were also been reported to produce antimicrobial peptides such as  $\beta$ -defensin and cathelicidin, which inhibit bacterial replication (Chen et al., 2009). In addition, basophils were the major sources of IL-4 in murine models of respiratory syncytial virus infection (Moore et al., 2009). The role of basophils in the protective immunity to bacterial or viral infections remains, however, relatively understudied. Altogether these data clearly suggest a crucial role for basophils in protective immunity during repeated infections.

## **9. Basophils in pathological conditions**

Apart from performing innate immune functions, basophils are known to regulate T and B cell responses. Therefore, an excessive basophils response could directly mediate heightened and inappropriate adaptive immune responses and consequences might lead to pathological conditions including allergy, autoimmune and inflammatory diseases.

### **9.1 *Basophils in allergic conditions***

Basophils can readily respond to variety of stimuli including IgE –allergen complexes, IL-3, IL-33 and TSLP. All these stimulations either alone or in a synergistic manner could induce release of Th2-promoting cytokines such as IL-4, IL-13 and TSLP. Further, basophils are the initial source of IL-4 and hence, might promote establishment as well as amplification of Th2 microenvironment in allergic diseases. Basophils also elicit symptoms of severe systemic anaphylaxis by secreting PAF upon binding of IgG-allergen immune complexes (Mukai et al., 2009) (Karasuyama et al., 2011). Further, IgE-allergen- mediated basophil responses are marked by the release of other vasoactive components such as histamine and leukotriene leading to vasodilation and bronchial constrictions that additionally intensify allergic symptoms. Basophils are reported to initiate and promote chronic allergic inflammation by IgE-mediated responses by inducing massive infiltration of eosinophils to the skin, in a mast cell and T cell-independent manner (Mukai et al., 2009). Increased numbers as well as infiltration of basophils were also been reported in the inflamed tissue of several allergic diseases such as allergic rhinitis, atopic dermatitis, urticaria and asthma (Ito et al., 2011) (Jeffery and Haahtela, 2006) (Kepley et al., 2001) (Schroeder, 2011). Basophils express several

chemokine receptors including CCR3, CXCR1, CXCR4 (Marone et al., 2005) (Ugucioni et al., 1997) and hence could migrate to site of inflammation upon signaling by diverse chemokines produced at inflammatory site. Anaphylatoxins such as C3a and C5a were also reported to act as effective chemo-attractants for basophils and could induce inflammatory mediators from IL-3-primed basophils (Marone, 2008) (Bischoff et al., 1990).

In addition, basophils play a critical role in allergy by modulating antibody production from B cells either indirectly by establishing Th2 environment or by directly inducing IgE synthesis from B cells through the expression of CD40L and production of cytokines IL-4, IL-6 and IL-13 (Marone et al. 2008).

## ***9.2 Basophils in the pathogenesis of autoimmune and inflammatory diseases***

The pathogenic role of basophils in autoimmune and inflammatory diseases are mainly accounted for the role of basophils in the polarization of Th2 responses by providing IL-4, IL-13 and TSLP and enhancing humoral responses by promoting differentiation and activation and survival of B cells and via IL-6 and BAFF (Karasuyama et al., 2011) (Kaveri et al., 2010). Recent report also indicated that basophils have a role in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) and lupus nephritis (Charles et al., 2010).

Mice lacking Src family protein tyrosine kinase Lyn (Lyn<sup>-/-</sup> mice) exhibit Th2 skewing in early life and develop autoimmune conditions in late phase. This autoimmune disease shared features with SLE such as presence of anti-double-stranded DNA antibodies and deposition of immune complexes in glomeruli accompanied by kidney damage (Charles

et al., 2010). Studies in mice deficient in both IgE and Lyn ( $Igh-7^{-/-};Lyn^{-/-}$ ), both IL-4 and Lyn ( $Il4^{-/-};Lyn^{-/-}$ ) revealed that IgE and IL-4 were majorly accounted for the development of lupus-like nephritis in  $Lyn^{-/-}$  mice and hence, signified the role of Th2 environment in the disease pathogenesis.

Implication of Th2 environment indicated likely association of basophil or mast cells in the development of autoimmune condition in  $Lyn^{-/-}$  mice. However, the Charles et al., observed marked reduction in numbers of circulating autoreactive IgGs, splenic plasma cells and in pro-inflammatory environment in kidneys upon basophil depletion. No alternation in disease process was observed in  $Kit^{W-sh/W-sh};Lyn^{-/-}$  mice that also lacked mast cells (Charles et al., 2010).

Basophils are known to possess FcεRI, high-affinity receptor for IgE and the binding of FcεRI to antigens or immune complexes could lead to basophil activation. Therefore, Charles et al., analyzed the levels of IgE-immune complexes in  $Lyn^{-/-}$  mice. They found high level of circulating IgE-immune complexes and increased sensitivity of basophils for IgE- immune complex-mediated activation of basophils in  $Lyn^{-/-}$  mice (Charles et al., 2010). Further, to explore if basophils could directly influence T and B cell responses, their lymph node homing potential was examined. Circulating basophils from  $Lyn^{-/-}$  mice showed increased expression of CD62L, the L-selectin that facilitates lymph node homing. This finding was further substantiated by observation of high levels of basophils and increased expression of MHC-II and membrane bound BAFF on basophils in the lymph nodes and spleen of  $Lyn^{-/-}$  mice (Charles et al., 2010). These observations demonstrated the capability of basophils to recruit to lymph nodes and spleen, which allows their interaction with T and B cells. Therefore, upon migration to lymphoid

organs, basophils could induce Th2 responses by expressing IL-4 and could also promote B cell survival, differentiation and auto-antibody production by expressing BAFF. This probable mechanism of disease progression was also testified in cohort of patients with SLE where elevated basophils, serum levels of auto-antibodies, IgE and anti-double-stranded DNA IgE-immune complexes were found. Levels of these self-reactive antibodies and immune complexes were positively correlated with disease activity and development of nephritis. Altogether, these results demonstrated active implication of basophils in the development and progression of autoimmune disease.

Another recent study revealed the association of basophils in eosinophilic esophagitis in an IgE independent manner. Artis and colleagues developed mice model of EoE (eosinophilic esophagitis), inflammatory disease associated with food allergy and is characterized by the accumulation of eosinophils, development of inflammation in esophageal tissues and increased expression of genes associated with Th2 responses (Noti et al., 2013). Owing to the association of EoE to polymorphisms in the gene encoding TSLP, the authors explored the role of TSLP in disease progression of EoE (Noti et al., 2013). By Blocking TSLP-TSLP-receptor (TSLPR) interaction using either TSLPR-deficient mice or antibody-mediated approach to neutralize TSLP in TSLPR-positive mice, the authors first established that TSLP-TSLPR interactions are necessary and sufficient for the development of experimental EoE-like disease, upon allergen challenge (Noti et al., 2013). TSLP-TSLPR interaction is often associated with IgE production and further development of allergic inflammation. Therefore, implication IgE in the development of EoE-like disease was explored by using IgE-sufficient (*Igh-7<sup>+/+</sup>*) and IgE-deficient (*Igh-7<sup>-/-</sup>*) mice. Consistent with clinical observation where IgE-specific

mAb failed to ameliorate EoE in patients, EoE-like disease was equally developed in *Igh-7<sup>+/+</sup>* and *Igh-7<sup>-/-</sup>* mice, confirming IgE-independent mechanism involved in the development of EoE (Noti et al., 2013).

Since TSLP is reported to elicit selective expansion of basophils (Siracusa et al., 2011), role of basophils in TSLP-mediated development of EoE-like disease in mice was explored. Interestingly, reduced accumulation of eosinophils in esophagus along with reduced expression of genes associated with Th2 cytokine responses was observed upon *in vivo* depletion of basophils. Consistent with these results, significant abrogation in the inflammation was seen upon TSLP neutralization in the established experimental EoE-like disease (Noti et al., 2013). These results showed that basophils and TSLP might be the significant contributors in the pathogenesis of experimental EoE-like disease.

To testify implication of TSLP-basophil axis in clinical settings, esophageal biopsies from a cohort of pediatric as well as adult subjects were examined. Enhanced expression of *TSLP* along with increased frequency of basophils and eosinophils was found in the esophageal biopsy of patients with active EoE as compared to healthy and inactive EoE subjects (Noti et al., 2013). Therefore, the authors described a genetic link between a gain-of-function polymorphism in TSLP and amplified responses of basophils.

Basophils also have an ability to migrate to mucosal and skin surfaces and are detected in the skin lesions of various inflammatory and autoimmune skin diseases as reported in atopic dermatitis, prurigo, urticaria, bullous pemphigoid, eosinophilic pustular folliculitis, Henoch-Schönlein purpura and dermatomyositis (Ito et al., 2011). Th2-type cytokines IL-4 and IL-13 are known to induce dermal fibroblasts to produce CCL11 and CCL26, which upon binding to CCR3 expressed by basophils, could facilitate basophil migration.

Therefore, the recruitment of basophils to site of lesion would further enhance Th2 environment. Also, circulating basophils from patients with inflammatory skin diseases such as in urticaria are found to be activated as analyzed by surface expression of CD203c as well as assessed by hyper-responsiveness for IL-3 and enhanced histamine release upon surface IgE cross-linking (Lourenco et al., 2008) (Grattan et al., 1991) (Bischoff et al., 1996). The factors responsible for activated phenotype of basophils in urticaria patients are not completely known. However, treatment with sera from patients with chronic idiopathic urticaria showed increased expression of CD203c on basophils (Yasnowsky et al., 2006), suggesting the presence of basophil stimulating factor(s) in the sera. However, depletion of IgG in the representative sera from chronic idiopathic urticaria patients showed significant decrease in CD203c expression on basophils. These results are consistent with the fact that functional IgG antibody to the  $\alpha$  subunit of the Fc $\epsilon$ RI or to IgE which trigger activation of basophils, was found in about 30-40% patients with chronic idiopathic urticaria (autoimmune urticaria). Further, increased plasma level of histamine altogether with increased ability of basophils to release histamine as reported systemic sclerosis patients, indicates implication of basophils in the pathogenesis of systemic sclerosis (de Paulis et al., 1991). Therefore, these reports provide an insight on the role of basophils in the pathogenesis of various inflammatory and autoimmune conditions beyond allergic diseases.

Noticeably, Chen et al., demonstrated IgD-mediated production of antimicrobial, pro-inflammatory and B cell-stimulating factors from basophils. Circulating IgD has been reported to interact with basophils through an unidentified calcium-fluxing receptor and subsequent cross-linking of surface-bound IgD could activate basophils. Of note, IgE –

activated basophils exhibit B cell activation functions mostly via CD40L, whereas IgD-crosslinking on basophils induce B cell activation and survival via IL-4, IL-13, BAFF and APRIL (Chen et al., 2009).

Patients with hyper-IgD syndrome (HIDS) such as TNF receptor-associated periodic fever syndrome (TRAPS), Muckle-Wells syndrome (MWS) and periodic fever-aphthous stomatitis-pharyngitis-cervical adenitis (PFAPA) syndrome had less circulatory and more mucosal IgD-armed basophils, indicating IgD-mediated induced mucosal homing and proliferation of basophils. These mucosal basophils also showed signs of hyper-activation based on strong BAFF, APRIL and LL-37 (cathelicidin) expression (Chen et al., 2009), indicating implication of basophils in B cells activation and inflammatory responses. Further, in healthy subjects, IL-3 treated basophils are also reported to secrete pro-inflammatory IL-1 $\beta$  and TNF upon IgD cross-linking and not by IgE-crosslinking (Chen et al., 2009).

## 10. Therapeutic approaches to target basophils

Considering the implication of basophils in several allergic, inflammatory and autoimmune diseases, development of therapeutic strategies to target basophils is essential. The use of anti-inflammatory agents has been the most commonly used strategy to suppress basophil activation and release of inflammatory mediators. Commonly used anti-inflammatory agents include glucocorticosteroids, tyrosine kinase and phosphatidylinositol 3-kinase inhibitors, methotrexate and histamine H1-antagonist desethoxycarbonyl-loratadine. These agents inhibit the IgE-dependent release of histamine by basophils (Schleimer et al., 1982) (Kleine-Tebbe et al., 1994) (Tedeschi et al., 2000). Advances in biotechnology, protein engineering and knowledge on basophils with reference to phenotype and signaling cascades, lead to the identification of novel protein/antibody-based therapeutic molecules. For example, **Omalizumab**, a monoclonal anti-IgE antibody has been reported to lower the levels of circulating IgE and to decrease the expression of FcεRI on basophils, and therefore, found to be effective for the treatment of allergic respiratory disorders (Lin et al., 2004). Recently it was described that among human circulating cells, basophils express the highest levels of inhibitory IgG receptor FcγRIIB (Tam et al., 2004) (Cassard et al., 2012). Studies demonstrated that co-engagement of FcεRI with FcγRIIB results in FcγRIIB-dependent inhibition of IgE-induced responses of both human and mouse basophils (Cassard et al., 2012) (Tam et al., 2004). It was also reported that these inhibitory signals generally dominate over activating signals when basophils encounter IgG-immune complexes, suggesting a tightly regulated

mechanism to prevent activation of basophils by circulating IgG-immune complexes.

These notions are exploited in several complementary approaches:

i) Human bifunctional  $\gamma$  and  $\epsilon$  heavy chain (Fc $\gamma$ -Fc $\epsilon$ ) fusion protein GE2. This fusion protein has been shown to inhibit allergic reactivity by inhibiting Fc $\epsilon$ RI-mediated degranulation (Zhu et al., 2002), by cross-linking Fc $\epsilon$ RI with Fc $\gamma$ RIIB.

ii) Monovalent bispecific anti-Fc $\gamma$ RII/anti-IgE antibody. This antibody was shown to co-aggregate Fc $\epsilon$ RI with Fc $\gamma$ RII on human basophils and prevented histamine release following triggering of antigen-specific IgE-sensitized cells by antigen (Zhu et al., 2002).

iii) 2G10, a chimeric human IgG1 anti-idiotypic. 2G10 simultaneously recognizes Fc $\epsilon$ RI-bound IgE via its Fab portions, and inhibitory Fc $\gamma$ RIIB, via its Fc portion. This antibody has been shown to inhibit both anti-IgE and allergen-driven basophil degranulation (Wigginton et al., 2008).

iv) Bispecific fusion protein anti-IgE DARPin-Fc. This fusion protein efficiently co-engages Fc $\epsilon$ RI-bound IgE with inhibitory Fc $\gamma$ RIIB on the surface of basophils to prevent pro-inflammatory mediators release by these cells (Zhu et al., 2002).

## **Objectives**

Basophils are rare circulating granulocytes and have long been studied for their role in allergic and hypersensitive reactions. Several reports in the past few years have revealed novel roles of basophils in the regulation of immune responses. Studies in murine models indicated that basophils could skew CD4<sup>+</sup> T cell responses towards Th2 phenotype, by providing IL-4, IL-13 and TSLP. Basophils were also demonstrated to modulate humoral responses by regulating B cell activation, proliferation and antibody production by providing of IL-6 and BAFF. However, most of these attributes have only been shown in the mice but not in the human system. Paucity of basophils in blood and difficult isolation procedures, make them less accessible for the investigations. Consequently, many aspects of basophils biology are still unexplored in humans. Therefore, I focused my thesis on two major aspects of human basophils.

### **Objective 1: The role of human basophils as APC in driving CD4<sup>+</sup> T cell responses**

Inability of DCs to produce IL-4 and to phagocytose large multicellular helminths, questions the potential of DCs to mount Th2 responses. However, basophils were reported as initial source of IL-4 and could respond to allergens and helminth-derived protease antigens. In this regard, recent reports in mice demonstrated that basophils could directly function as APCs. By presenting antigens to T cells in the context of MHC-II and CD80/86, murine basophils mediate Th2 responses against protease allergen and helminth parasite infection.

However, validation of these reports in the context of human system is still pending. Thus, I investigated whether circulating human basophils have the capability to modulate CD4<sup>+</sup> T cell responses by acting as APCs.

Further, basophils are mostly been studied in the context of Th2 responses. However, owing to implication of basophils in several inflammatory and autoimmune diseases, impact of these cells in mediating other T cell responses such as Th17, is not much explored. Therefore, I sought to explore role of basophils as APC in mounting Th17 responses.

### **Objective 2: Regulation of basophil functions by CD4<sup>+</sup>CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs**

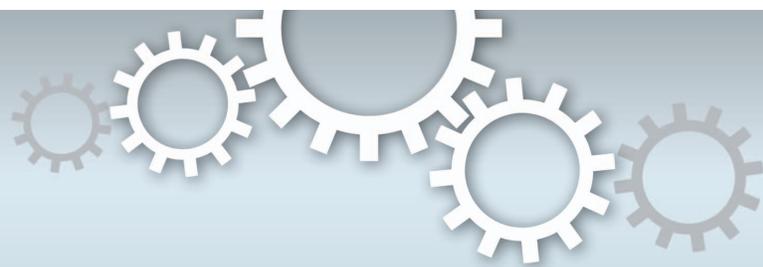
Basophils are reported to modulate T cell polarization and B cell functions and therefore are capable to skew the immune responses. Their dysregulated functions were found to be associated with development of autoimmune and inflammatory conditions such as lupus nephritis, urticaria and eosinophil esophagitis. However, it is not known how are the functions of basophils being regulated. As Tregs are known for their immune surveillance functions, I intended to investigate whether Tregs could modulate basophil activation and functions.

# Results

## **Article 1- Résumés en français**

### **Titre: Les basophiles circulants sont dépourvus de caractéristiques de cellules de présentatrices d'antigènes professionnelles**

Plusieurs études effectuées chez les souris ont montré que les basophiles possèdent des caractéristiques de cellules présentatrices d'antigènes professionnelles (CPA). Ils expriment les complexes majeurs d'histocompatibilité de classe II (CMH-II ou HLA-DR) ainsi que les marqueurs de co-stimulation CD86 et CD80. De plus, les basophiles murins capturent et présentent les antigènes solubles ou les complexes IgE-antigène et induisent une polarisation de type Th2 de la réponse immunitaire. Par conséquent, nous avons exploré la capacité des basophiles humains à agir comme CPA professionnelles. Nos résultats montrent que, contrairement aux cellules dendritiques ou des monocytes, les basophiles humains circulant non stimulés n'expriment pas à leur surface HLA-DR ou les marqueurs de co-stimulation CD80 et CD86. De plus, la stimulation des basophiles par divers allergène ne modifie pas l'expression de ces marqueurs. Pour valider l'expression de marqueurs, l'allergène Asp F1 soluble, un allergène d'*Aspergillus fumigatus*, Bet v 1, le majeur allergène des bouleaux, des ligands de TLR2 ou des IgE ont été utilisés. De plus, contrairement des cellules dendritiques, la co-culture des basophiles chargés par Asp f 1 avec des lymphocytes T CD4+ ne favorisent pas une réponse immunitaire de type Th2. La polarisation a été mesurée par la détection de la cytokine IL4 dans le surnageant. En conclusion, Nos résultats montrent que les basophiles circulants humains ne possèdent pas les fonctions de cellules présentatrices d'antigènes professionnelles.



# Circulating human basophils lack the features of professional antigen presenting cells

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Recent reports in mice demonstrate that basophils function as antigen presenting cells (APC). They express MHC class II and co-stimulatory molecules CD80 and CD86, capture and present soluble antigens or IgE-antigen complexes and polarize Th2 responses. Therefore, we explored whether human circulating basophils possess the features of professional APC. We found that unlike dendritic cells (DC) and monocytes, steady-state circulating human basophils did not express HLA-DR and co-stimulatory molecules CD80 and CD86. Basophils remained negative for these molecules following stimulation with soluble Asp f 1, one of the allergens of *Aspergillus fumigatus*; Bet v 1, the major birch allergen; TLR2-ligand or even upon IgE cross-linking. Unlike DC, Asp f 1-pulsed basophils did not promote Th2 responses as analyzed by the secretion of IL-4 in the basophil-CD4<sup>+</sup> T cell co-culture. Together, these results demonstrate the inability of circulating human basophils to function as professional APC.

Basophils are the rare granulocytes and represent < 1% of circulating leukocytes. They are FcεRI<sup>+</sup> CD203c<sup>+</sup> CD123<sup>+</sup> and play an important role in the protection against helminth parasites and in the pathogenesis of allergic diseases and in autoimmune conditions such as lupus nephritis and rheumatoid arthritis<sup>1-7</sup>. Basophils are activated by recognition of protein allergens, proteases and helminth parasite antigens via pattern recognition receptors and FcεRI-mediated signaling. The latter is initiated by cross-linking of FcεRI either through binding of allergen to surface-bound IgE or by IgE-immune complexes. All these events lead to the secretion of IL-4, IL-13, B cell-activating factor belonging to the TNF family (BAFF) and thymic stromal lymphopoietin (TSLP) that help in the programming of Th2 responses and stimulation of antibody production by B cells<sup>3,8-10</sup>. In addition, basophils also release other inflammatory mediators such as histamine and leukotrienes that mediate vasodilation and infiltration of immune cells.

Recent reports have shown that basophils could also act as professional antigen presenting cells (APC) in various murine models<sup>8,11-14</sup>. Professional APC are those that can uptake the antigens, process and present the antigenic peptides in the context of MHC molecules and co-stimulatory molecules to CD4<sup>+</sup> T cells leading to activation, proliferation and polarization of CD4<sup>+</sup> T cell responses<sup>15</sup>. Until now, dendritic cells (DC), macrophages/monocytes and memory B cells were considered as professional APC. However, recent studies in mice have demonstrated that basophils express MHC class II and co-stimulatory molecules CD80 and CD86, capture and present soluble antigens or IgE-antigen complexes and polarize Th2 responses<sup>11-13</sup>. Therefore, in the present report, we explored whether circulating human basophils from healthy individuals possess the features of professional APC and mediate Th2 responses to soluble antigens.

## Results

**Circulating human basophils do not express HLA-DR and co-stimulatory molecules CD80 and CD86.** We first analyzed the expression of antigen presenting molecule HLA-DR and co-stimulatory molecules of B7 family

SUBJECT AREAS:  
GRANULOCYTES  
ANTIGEN-PRESENTING CELLS  
FUNGAL HOST RESPONSE  
DENDRITIC CELLS

Received  
10 December 2012

Accepted  
10 January 2013

Published  
1 February 2013

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in the steady-state basophils. Previous reports have demonstrated that circulating basophils from naïve mice express detectable levels of these molecules<sup>13</sup>. However, we could not detect the expression of either HLA-DR or co-stimulatory molecules CD80 and CD86 on the circulating basophils (Fig. 1). The expression of these molecules was less than 1%. On the contrary, 100% of steady-state circulating monocytes and monocyte-derived DC expressed HLA-DR molecules. Although monocytes did not express CD80, nearly 80% DC expressed this molecule. Further, CD86 was expressed both on monocytes (about 90%) and on DC (about 30%) (Fig. 1).

The lack of HLA-DR expression on purified basophils was not due to depletion of these cells during purification process by basophil

isolation kit II (Miltenyi Biotech) that contained anti-HLA-DR antibodies in the antibody cocktail. Analysis of basophils in the blood even before purification revealed a lack of HLA-DR expression (Fig. 2a). In addition, the expression level of HLA-DR was  $\leq 1\%$  on the basophils isolated by EasySep Human Basophil enrichment kit (Stem cell technologies) that did not deplete HLA-DR-expressing basophils (Fig. 2b).

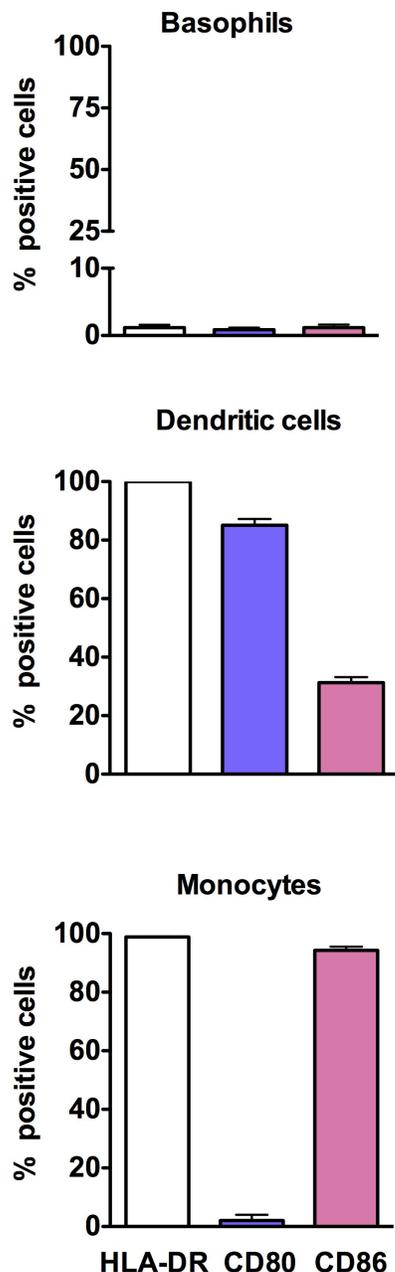
**Dendritic cells but not basophils show enhanced expressions of HLA-DR, CD80 and CD86 upon interaction with allergens.** When APC are stimulated with antigens, these cells undergo activation and the process is characterized by the enhanced expression of antigen-presenting and co-stimulatory molecules. Therefore we investigated whether interaction of circulating human basophils with allergens leads to induction of HLA-DR, CD80 and CD86 on the cells. We found that stimulation of DC with Asp f 1, one of the major allergens of *Aspergillus fumigatus*, lead to increased expression of HLA-DR and co-stimulatory molecules (Fig. 3a). On the contrary, basophils remained negative for these molecules (Fig. 3b). As basophils express toll-like receptor 2 (TLR2)<sup>16</sup>, we further examined the expression of HLA-DR, CD80 and CD86 on basophils stimulated with TLR2-ligand. However, we did not observe the expression of above molecules on TLR2-stimulated basophils (Fig. 3c and 3d).

We confirmed that basophils were viable and fully functional after stimulation with Asp f 1. The level of apoptosis in IL-3-treated basophils was in the range of 2.3 to 6.2% (Fig. 4). Compared to this, apoptosis in Asp f 1-treated basophils was in the range of 1.8 to 4.8% (Fig. 4). Thus, the level of apoptosis in control and Asp f 1-treated basophils did not differ significantly. Further, Asp f 1-treated basophils were functionally viable as determined by the activation status of cells. The levels of expression of activation-associated markers CD63, CD203c and CD13 were similar both in control and in Asp f 1-stimulated basophils upon anti-IgE-stimulation (Fig. 5).

Further, we investigated whether the lack of expression of antigen presenting and co-stimulatory molecules on basophils was restricted to only to Asp f 1 or to other allergens. We analyzed the expression of HLA-DR and co-stimulatory molecules on the basophils stimulated with other well-defined allergens such as Bet v 1, the major birch pollen allergen and Phl p 5, a major timothy grass pollen allergen. The level of apoptosis in Bet v 1-treated basophils was in a similar range as that of IL-3-treated control basophils (Fig. 4). Also, we did not observe the expression of HLA-DR, CD80 and CD86 on basophils upon stimulation with these allergens (Fig. 6 and data not shown). Similar results were also observed with other antigens of *A. fumigatus*, superoxide dismutase (Sod1p) and mycelial catalase 1 (Cat1p) (data not shown).

In the context of secondary immune responses, IgE-bound allergens could trigger basophil activation. Therefore, to mimic this situation and to demonstrate unequivocally that the lack of HLA DR, CD80 and CD86 expression on basophils were not restricted to stimulation of cells with a given antigen, we stimulated basophils by IgE crosslinking. However, we could not observe the expression of these molecules on basophils under this stimulation condition (Fig. 6). Together these results indicated that although basophils from mice do express antigen presenting and co-stimulatory molecules, circulating human basophils do not express these molecules under any stimulatory conditions.

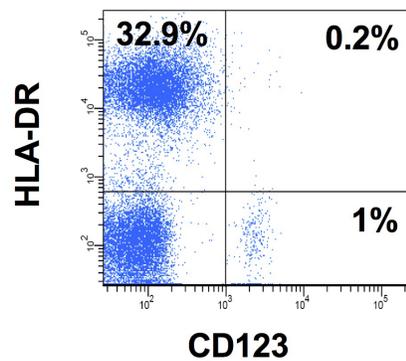
**Asp f 1-primed dendritic cells but not basophils promote Th2 responses.** One of the key functions of APC is to promote polarized T cell responses. Asp f 1-primed DC or basophils were co-cultured with autologous CD4<sup>+</sup> T cells at 1:40 ratio and the Th1 and Th2 responses were measured by analyzing the quantities of IFN- $\gamma$  and IL-4 in the culture supernatants. We show that, DC that were primed with Asp f 1, increased the production of IL-4 from CD4<sup>+</sup> T cells thus indicated the promotion of Th2 responses by Asp f 1-primed DC (Fig. 7a). However, Asp f 1-pulsed basophils did not



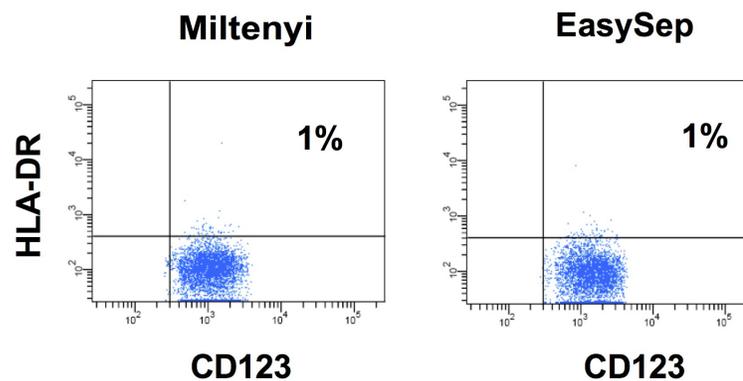
**Figure 1** | Expression of HLA-DR and co-stimulatory molecules CD80 and CD86 on circulating human basophils, monocytes and monocyte-derived DC. The expression of markers was determined by flow cytometry by using fluorochrome-conjugated monoclonal antibodies. The results are presented as percentage positive cells. Data (mean  $\pm$  SEM) are from three independent donors.



a



b



**Figure 2 | Expression of HLA-DR on basophils in the circulation and in the cells isolated by two different kits. (a)** Red blood cells in the buffy bags were lysed and stained for HLA-DR and CD123 (b) Basophils were isolated either by EasySep Human Basophil enrichment kit that does not deplete HLA-DR expressing basophils or by basophil isolation kit II. The expression of HLA-DR and CD123 was analyzed by flow cytometry.

mount Th2 responses. The level of IL-4 secretion was on par with CD4<sup>+</sup> T cells co-cultured with unstimulated basophils or Asp f 1-stimulated CD4<sup>+</sup> T cells (Fig. 7a). The IFN- $\gamma$  production from CD4<sup>+</sup> T cells remained unchanged both by DC and basophils (Fig. 7b). Together, these results confirmed the inability of circulating human basophils to function as professional APC.

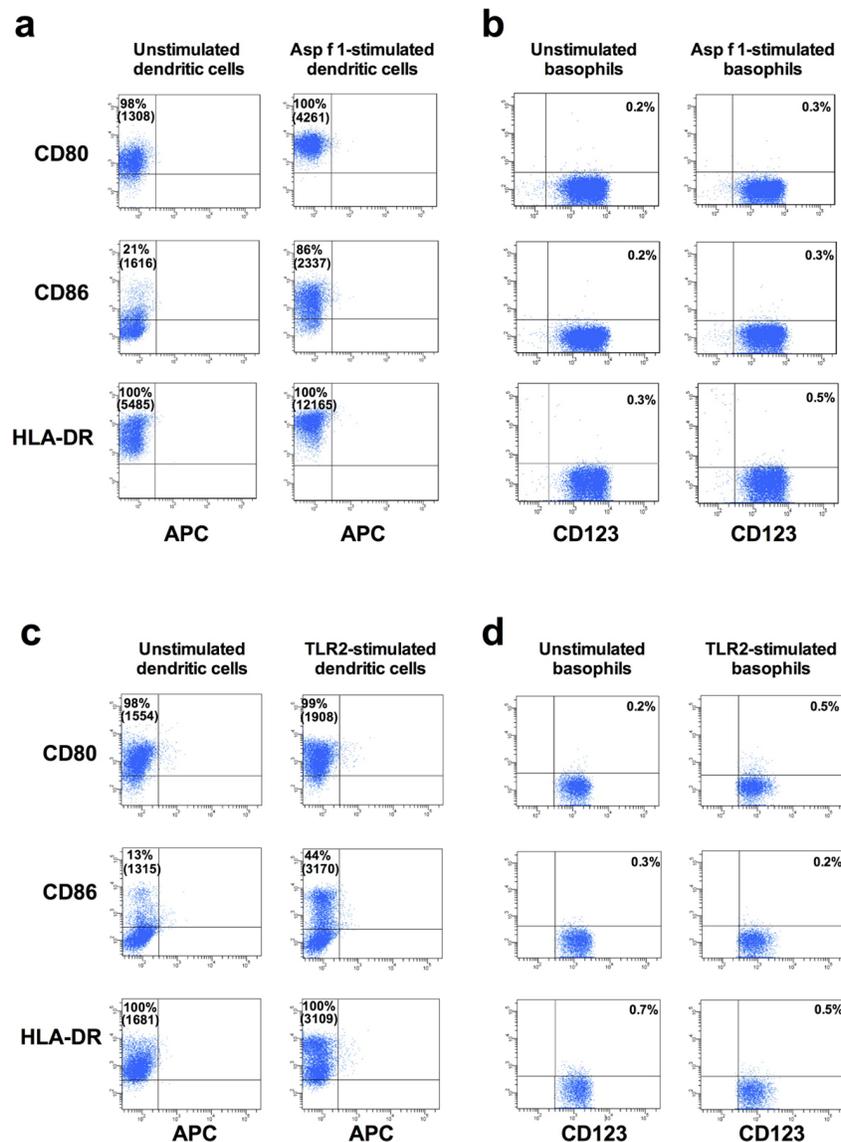
## Discussion

The main function of professional APC is to present the antigen to CD4<sup>+</sup> T cells and to polarize T cell responses at low APC : CD4<sup>+</sup> T cell ratios<sup>15</sup>. APC-mediated CD4<sup>+</sup> T cell polarization implicates four different signals: signal zero at the interface of APC and the antigen, signal 1 at the interface of antigen-loaded HLA-DR on APC with T cell receptor-CD3 complexes on T cells, signal 2 that implicates the interaction of co-stimulatory molecules CD80/CD86 on APC with CD28 of T cells and signal 3 in the form of CD4<sup>+</sup> T cell-polarizing cytokines secreted by APC. Although, human basophils secrete high amounts of Th2-polarizing cytokine IL-4, our results demonstrate that unlike other professional APC, circulating human basophils lack signal 1 and signal 2 that are implicated in T cell polarization.

Previous studies by using basophils from allergic patients have demonstrated that human basophils do not internalize and present IgE-bound antigens<sup>17,18</sup>. These reports however studied the function of basophils in the context of secondary immune responses and did

not confirm whether human circulating basophils could present soluble antigens in the context of primary immune responses to polarize T cell responses. In addition, whether direct stimulation of circulating human basophils with an allergen could lead to expression of antigen presenting and co-stimulatory molecules was also not addressed. Professional APC could internalize not only immune complexes but also soluble antigens via receptor-mediated endocytosis, pinocytosis and phagocytosis<sup>19</sup>. The signaling via antigens (such as pathogen-associated molecular patterns) leads to activation of APC with enhanced expression of antigen-presenting molecules and co-stimulatory molecules. Basophils did not express these molecules despite priming with Asp f 1, Bet v 1 and other allergens. As a consequence, unlike DC, basophils did not polarize Th2 responses (as measured by IL-4). Even at higher ratios of basophils : CD4<sup>+</sup> T cells (1 : 10) there were no apparent polarization of Th2 responses. On the other hand, DC were activated by Asp f 1 and induced Th2 polarization. These results thus confirmed the previous reports that DC could mediate allergic immune responses upon interaction with allergens<sup>20</sup>.

*A. fumigatus* is one of the major fungal species implicated in the pathogenesis of several respiratory diseases including asthma and allergic broncho-pulmonary aspergillosis (ABPA)<sup>21</sup>. Asp f 1 is a 18 kd ribonuclease protein of ribotoxin family and is one of the major allergens of *Aspergillus*. Presence of IgE-reacting to Asp f 1 is a



**Figure 3 | Monocyte-derived DC but not basophils show enhanced expressions of HLA-DR, CD80 and CD86 upon stimulation with Asp f 1 and peptidoglycan.** (a and c) Monocyte-derived DC were cultured in X-Vivo medium containing 5% human AB serum and cytokines GM-CSF and IL-4 (panel: unstimulated dendritic cells) or cytokines plus Asp f 1 (panel: Asp f 1-stimulated dendritic cells) or cytokines plus peptidoglycan (panel: TLR2-stimulated dendritic cells) for 24 hours. The phenotype of DC was analyzed by flow cytometry. (b and d) Basophils were cultured in X-Vivo medium containing 5% human AB serum and IL-3 alone (panel: unstimulated basophils) or IL-3 plus Asp f 1 (panel: Asp f 1-stimulated basophils) or IL-3 plus peptidoglycan (panel: TLR2-stimulated basophils) for 24 hours. The phenotype of basophils was analyzed by flow cytometry. Percentage of cells expressing indicated markers and mean fluorescence intensity values (in parenthesis) are indicated. Results are representative of at least three experiments.

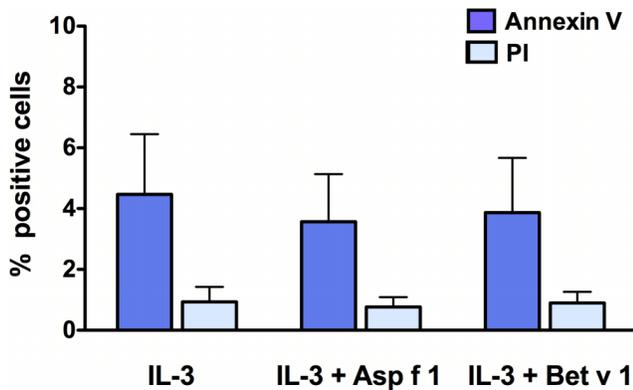
common feature of ABPA and allergic asthma<sup>22,23</sup>. Previous studies have also demonstrated that Asp f 1 is highly immunogenic and could activate APC such as DC to induce maturation of cells as well as pro-inflammatory cytokine and chemokine responses<sup>24,25</sup>. However, in the air-borne dormant conidia, Asp f 1 is covered by hydrophobin layer that masks Asp f 1 being recognized by innate cells<sup>24,26</sup>.

However, our present results demonstrate that Asp f 1 could not be presented by circulating human basophils to initiate immune responses. As Asp f 1-pulsed DC promoted Th2 responses, these results indicate that IgE antibody responses to Asp f 1 in ABPA and allergic asthma patients could be mediated mainly via DC. Also data from experimental models (DC vaccination model) have shown that Asp f 1 induces Th2 responses<sup>27</sup>.

Protease allergens could trigger innate responses including DC via protease-activated receptors (PARs)<sup>28–30</sup> and fungal proteases

were proposed to initiate inflammatory responses via PARs<sup>31–33</sup>. Therefore, based on these data, it is likely that Asp f 1 could activate DC via PAR signaling<sup>34</sup>. However, PARs were found to be absent on human basophils<sup>35</sup> and hence potential innate trigger for protease allergens on basophils is still a mystery. However, in the context of secondary immune responses, IgE-bound allergens could trigger basophil activation. But, we did not observe the expression of antigen presenting and co-stimulatory molecules on basophils even upon IgE crosslinking. Similarly, TLR2 stimulation did not induce expression of HLA-DR, CD86 and CD80 on basophils.

We used percoll density gradient of three different densities (1.070, 1.079, 1.088) for obtaining basophil rich peripheral blood mononuclear cells (PBMC)<sup>36</sup> and bead-based negative isolation method to isolate circulating human basophils from PBMC. Basophils in the circulation were in the range of 0.4 to 1% and in



**Figure 4 | The viability of basophils.** Basophils were cultured in complete medium containing IL-3 alone or with Asp f 1 or Bet v 1 for 24 hours. Viability of basophils was analyzed by surface expression of apoptotic maker, Annexin V and necrotic marker, propidium iodide (PI). Data (mean  $\pm$  SEM) are from three independent experiments.

the percoll purified PBMC, they were in the range of 0.5 to 1.2%. The recovery of basophils by using negative selection on a MACS system was in the range of 0.3–1.5%. However, the yield was lower with EasySep Human Basophil enrichment kit (0.05–0.2%). The purity of isolated basophil population was generally  $> 95\%$  as analyzed by combination of various markers such as CD203c and CD123. There were no contaminating CD14<sup>+</sup> cells in the isolated populations. Also, the lack of polarization of CD4<sup>+</sup> T cells in the co-culture of CD4<sup>+</sup> T cells with Asp f 1-primed basophils, indirectly ruled out the presence of any contaminating APC in the basophil population.

Most of the results in murine models come from basophils isolated from either lymph nodes or spleen following antigen stimulation<sup>11–13</sup>. Although basophils are circulating cells and normally not tissue resident, it is not known whether migration of human basophils to lymphoid tissues could alter the phenotype and expression of antigen presenting and co-stimulatory molecules. Therefore, further studies on lymphoid tissue basophils are required to support our observations.

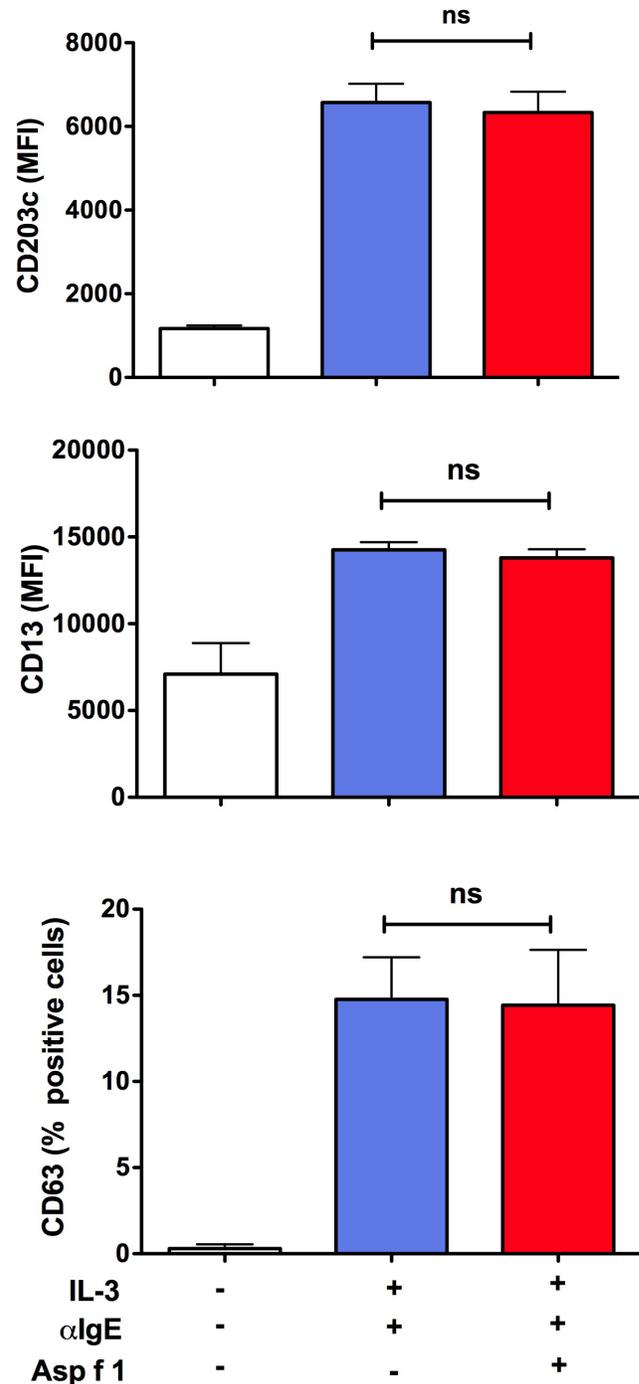
## Methods

**Isolation of circulating human basophils, monocytes and generation of monocyte-derived DC.** Buffy bags from the healthy donors were obtained from Hôpital Hôtel Dieu, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors. Basophil-rich fraction of PBMC was obtained by percoll density gradient centrifugation<sup>36</sup>. Basophils from these basophil-rich PBMC were isolated either by basophil isolation kit II (Miltenyi Biotec, Paris, France) or EasySep human basophils enrichment kit (Stem cell technologies, Grenoble, France). Monocytes were isolated from PBMC by using CD14 magnetic micro-beads (Miltenyi Biotec). The monocytes were cultured in GM-CSF (1000 IU/10<sup>6</sup> cells) and IL-4 (500 IU/10<sup>6</sup> cells) (both from Miltenyi Biotec) for five to six days to generate DC<sup>37</sup>.

**Stimulation of cells.** Immature DC were stimulated either with recombinant Asp f 1 (1  $\mu$ g/0.5  $\times$  10<sup>6</sup> cells) or with peptidoglycan (5  $\mu$ g/0.5  $\times$  10<sup>6</sup> cells) in the X-Vivo medium containing 5% AB serum, IL-4 and GM-CSF. The recombinant Asp f 1 protein (18-kDa RNase, Afu 5g02330) from *A. fumigatus* was produced as previously described<sup>38</sup>. The purity of protein was analyzed by SDS-PAGE followed by revealing the protein band by silver staining and MALDI-TOF mass spectrometry. After 24 hours stimulation, DC were washed two times with large amounts of RPMI 1640 to remove residual IL-4 and GM-CSF. The cells were then processed either for flow cytometry or co-culture with CD4<sup>+</sup> T cells.

Basophils were cultured in X-Vivo medium containing 5% human AB serum. IL-3 (100 ng/10<sup>6</sup> cells) was also added to the medium in order to maintain viability of the cells. The cells were stimulated for 24 hours with peptidoglycan (5  $\mu$ g/0.5  $\times$  10<sup>6</sup> cells), recombinant Asp f 1, Sod1p, Cat1p, Bet v 1, Phl p 5 (all at 1  $\mu$ g/0.5  $\times$  10<sup>6</sup> cells). Recombinant Sod1p (Afu 5g09240) and Cat1p (Afu 3g02270) from *A. fumigatus* were produced as previously described<sup>38</sup> and recombinant Bet v 1 and Phl p 5 were kind gift from ALK-Abello (Copenhagen, Denmark). The cells were then used either for flow cytometry or co-culture with CD4<sup>+</sup> T cells.

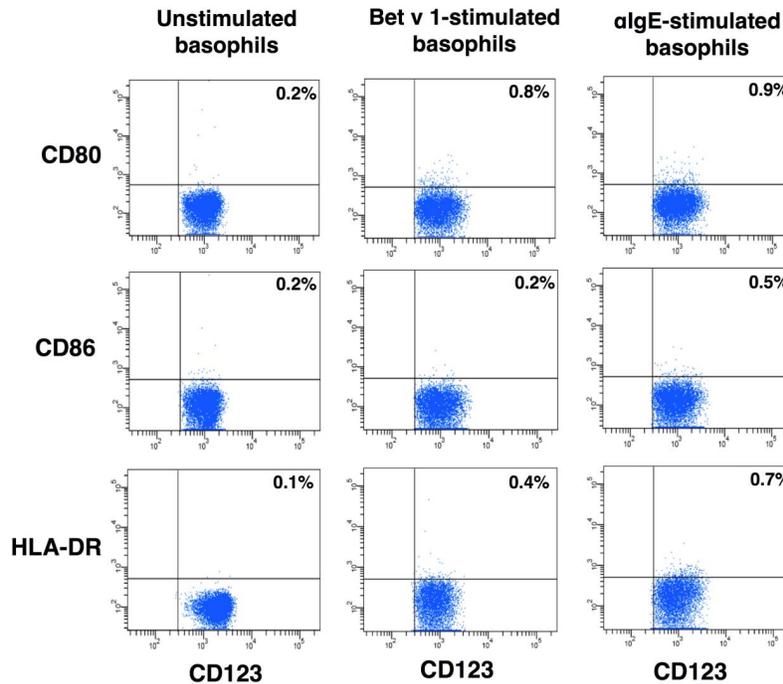
The viability of allergen-pulsed cells was analyzed by surface expression of apoptotic maker, Annexin V and necrotic marker, propidium iodide (PI). To determine



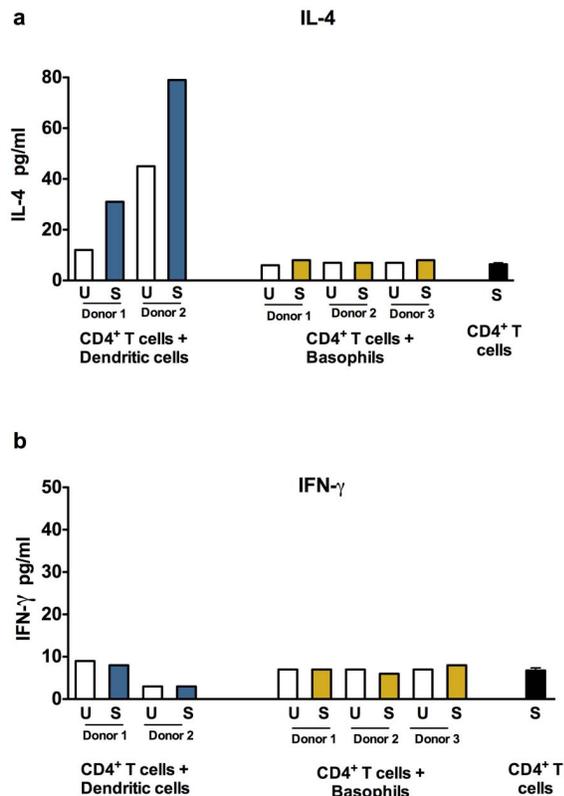
**Figure 5 | Asp f 1-treated basophils are functionally viable.** Basophils were cultured with or without Asp f 1 in the presence of IL-3 for 24 hours. These cells were stimulated with anti-IgE antibodies (10 ng/ml) during last one hour of the culture. Basophil response was determined by analyzing the expression of activation-associated markers CD203c, CD13 and CD63 by flow cytometry. Data (mean  $\pm$  SEM) are from three independent experiments.

whether Asp f 1-treated basophils were functionally viable, basophils were cultured with or without Asp f 1 in the presence of IL-3 for 24 hours. These cells were stimulated with anti-IgE antibodies (10 ng/ml) during last one hour of the culture. Basophil response was determined by analyzing the expression of activation-associated markers CD63, CD203c and CD13 by flow cytometry.

**Co-culture of basophils or DC with CD4<sup>+</sup> T cells.** Autologous CD4<sup>+</sup> T cells were negatively isolated from PBMC by using CD4<sup>+</sup> T cell isolation kit II from Miltenyi Biotec. The CD4<sup>+</sup> T cells (0.1  $\times$  10<sup>6</sup> cells/well in U-bottomed 96 wells plate) were



**Figure 6 | The lack of expression of antigen presenting and co-stimulatory molecules on basophils is a universal phenomenon.** Basophils were cultured in X-Vivo medium containing 5% human AB serum and IL-3 alone (panel: unstimulated basophils) or IL-3 plus Bet v 1 or IL-3 plus anti-IgE antibodies for 24 hours. The phenotype of basophils was analyzed by flow cytometry. Percentage of cells expressing indicated markers are indicated. Results are representative of three experiments.



**Figure 7 | Asp f 1-primed monocyte-derived dendritic cells but not basophils promote Th2 responses.** The autologous CD4<sup>+</sup> T cells were co-cultured with Asp f 1-pulsed (labeled as S) or unpulsed (labeled as U) basophils (Panel: CD4<sup>+</sup> T cells + Basophils) or monocyte-derived DC (Panel: CD4<sup>+</sup> T cells + Dendritic cells) at 40:1 ratio for 4 days in X-Vivo medium containing 5% human AB serum. The amount of IL-4 (a) and IFN-γ (b) in the cell-free culture supernatants was measured.

co-cultured with Asp f 1-pulsed or unpulsed basophils or DC at 40:1 ratio for 4 days in X-Vivo medium containing 5% human AB serum. The cell-free culture supernatants were used for measuring the T cell cytokines. Asp f 1-pulsed or unpulsed CD4<sup>+</sup> T cells (without DC or basophils) were used as controls.

**Flow cytometry.** The cell surface staining was performed by using fluorochrome-conjugated monoclonal antibodies to CD80, CD86, HLA-DR, CD14, CD63, CD13, Annexin V (all from BD Biosciences, Le Pont de Claix, France), CD203c (Beckman Coulter, Villepinte, France) and CD123 (Miltenyi Biotec). PI was purchased from Sigma-Aldrich (Lyon, France). The cells were then processed for flow cytometry (LSR II, BD Biosciences) and data were analyzed by BD FACS DIVA program.

**Cytokine analysis.** IFN-γ and IL-4 were measured in cell-free culture supernatants by using BD<sup>TM</sup> cytometric bead array human Th1/Th2 cytokine kit. The detection limits of IFN-γ and IL-4 were 7 pg/ml and 2.6 pg/ml respectively.

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## Acknowledgements

Supported by European Community's Seventh Framework Programme [FP7/2007–2013] under Grant Agreement No: 260338 ALLFUN and ANR-10-BLAN-1309 HYDROPHOBIN.

## Author contributions

J.B. designed research, M.S. & P.H. performed research, all authors analyzed the data, V.A., R.B., H.S., P.P. & J.-P.L. provided new research tools. J.B. wrote the paper and all authors reviewed and approved the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Sharma, M. *et al.* Circulating human basophils lack the features of professional antigen presenting cells. *Sci. Rep.* **3**, 1188; DOI:10.1038/srep01188 (2013).

## **Article 2,3,4 - Résumés en français**

### **Titre: Absence de polarisation des lymphocytes T mémoires CD4<sup>+</sup> en Th17 par les basophiles**

Les basophiles sont les granulocytes les plus rares. Ils jouent un rôle important dans la polarisation des réponses immunitaires de type Th2 et dans la protection contre les helminthes parasites. Des études récentes ont montré un rôle des basophiles dans la pathogénèse des plusieurs maladies inflammatoires et auto-immunes. De façon intéressante, d'autres études ont montré l'implication des cellules Th17 dans le développement des certaines de ces pathologies comme l'asthme, la sclérodermie systémique, l'urticaire chronique, le lupus, le purpura de Henoch-Schönlein et Pemphigoïde bulleuse. Dans ce travail, nous avons exploré le rôle des basophiles dans le développement de l'inflammation en favorisant l'expansion des lymphocytes T humain de type Th17. Nos résultats montrent l'absence de l'expansion de cellules Th17 par les basophiles et d'induire la synthèse et sécrétion des cytokines spécifiques des cellules Th17. L'absence de polarisation est observée par contact direct ou indirectement via des Cellules présentatrices d'antigènes professionnelles. Ainsi, nos résultats montrent que le rôle des basophiles dans les maladies auto-immunes et antiinflammatoires est indépendant de l'expansion de lymphocytes T de type Th17.

# Correspondence

## Mediation of T-Helper 17 Responses to Schistosomes by Dendritic Cells but Not Basophils

TO THE EDITOR—T-helper (Th) 17 cells that express lineage-specific transcription factor RORC and produce cytokines interleukin 17A (IL-17A), interleukin 17F (IL-17F), and interleukin 22 (IL-22), play a key role in the clearance of both intracellular and extracellular bacteria and fungal infections. However, in the context of certain other infections, such as hepatitis B, chronic toxoplasmic uveitis, encephalitis, and Theiler murine encephalomyelitis, chronic activation of Th17 cells could contribute to tissue damage and immunopathology [1–3]. In line with these observations, Mbow et al [4] recently demonstrated that Th17 cells are associated with pathology in human schistosomiasis. They found elevated Th17 cells in peripheral blood of patients with schistosomiasis as well as in the liver and spleen of *Schistosoma mansoni*-infected mice. However, considering the essential role of innate immune cells in mediating T-cell responses, questions regarding the identification of innate cells that could promote Th17 responses during *Schistosoma* infection remain unexplored.

In this regard, dendritic cells (DCs) are innate immune cells extensively studied for their role in antigen presentation and in the regulation of T-cell responses to pathogens. However, there is also growing evidence to implicate basophils in eliciting immune responses against helminth parasites including schistosomes. Basophils were recruited rapidly to the lymph nodes after exposure to *S. mansoni* eggs [5]. Because both DCs and basophils have important roles in

regulating immune responses to pathogens, we sought to explore which of the innate cells are implicated in driving Th17 responses to schistosomes, as reported by Mbow et al [4].

Dendritic cells were generated from peripheral blood monocytes, and basophils were isolated from peripheral blood mononuclear cells of healthy donors, as described elsewhere [6]. The *S. mansoni* eggs were obtained from 3–4 livers of golden hamsters infected with parasite cercarial larvae for 40 days [7]. The cell suspension was washed 3 times with Hank's balanced salt solution followed by collagenase B (100 µg/mL) treatment. The egg pellet was washed again with phosphate-buffered saline 3 times at 400 g for 1 minute. Eggs were counted, and the absence of contaminating hamster tissue fragments in the egg preparation was verified by microscopic analysis. CD4<sup>+</sup> T cells were isolated from peripheral blood mononuclear cells by negative selection using a CD4<sup>+</sup> T-cell isolation kit (Miltenyi Biotec). Regulatory T cells were removed by using CD25 microbeads (Miltenyi Biotec), and CD4<sup>+</sup>CD25<sup>-</sup> T cells were used for the experiments.

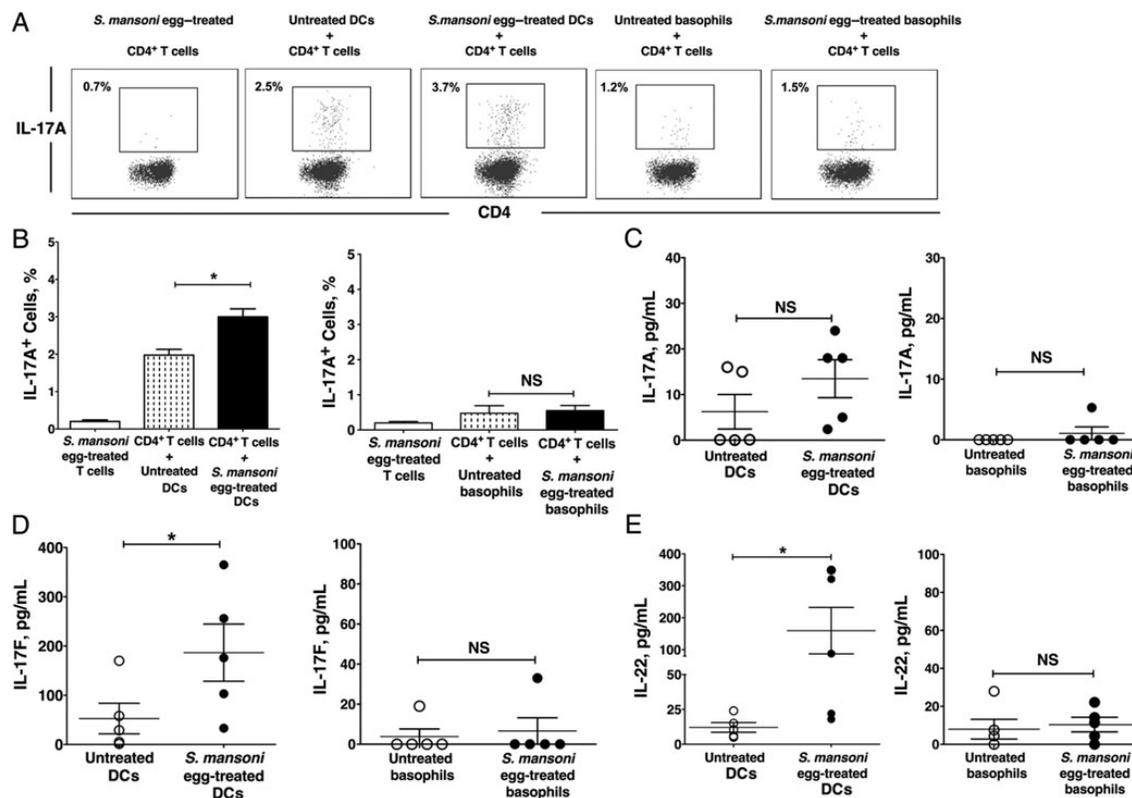
To investigate the differential effect of DCs and basophils to promote Th17 responses to schistosomes, either untreated or *S. mansoni* egg-treated DCs and basophils were cocultured with autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells. After 4 days of culture, Th17 responses were analyzed by means of flow cytometry (BD LSR II) and measurement of Th17 cytokines in the cell-free culture supernatants.

We found that *S. mansoni* egg-treated DCs promoted expansion of IL-17A<sup>+</sup> T cells in the mixed lymphocyte culture. The mean percentage (± standard error of the mean) of IL-17A<sup>+</sup> T cells in the

untreated DC–CD4<sup>+</sup>CD25<sup>-</sup> T-cell coculture was 1.9% ± 0.3% (n = 5) and was increased to 3% ± 0.5% (n = 5) by *S. mansoni* egg-treated DCs (Figure 1A and 1B). In contrast, *S. mansoni* egg-treated basophils did not promote Th17 cell expansion. The percentage of IL-17A<sup>+</sup> T cells in the CD4<sup>+</sup>CD25<sup>-</sup> T cells cocultured with *S. mansoni* egg-treated basophils was similar to that of untreated basophils (Figure 1A and 1B).

To corroborate these flow cytometric results, we analyzed the secretion of Th17-derived cytokines (IL-17A, IL-17F, and IL-22) in the cell-culture supernatants. Although the IL-17A<sup>+</sup> T-cell population was enhanced by *S. mansoni* egg-treated DCs, surprisingly, these CD4<sup>+</sup> T cells did not secrete high amounts of IL-17A (Figure 1C). On the contrary, *S. mansoni* egg-treated DCs significantly promoted the production of IL-17F as well as IL-22 from T cells in comparison with untreated DCs (Figure 1D and 1E). Of note, IL-17A and IL-17F are genetically linked; expression under the control of same locus, frequently coexpressed at the single cell level and have similar functions. Both IL-17A and IL-17F are critical for the recruitment of macrophages and neutrophils and act on a wide range of immune and nonimmune cells to induce diverse inflammatory cytokines and chemokines including tumor necrosis factor, interleukin 6, interleukin 1β, CXCL1, and CXCL8 [8]. Thus, our results indicated that the Th17 responses and pathogenesis of schistosomiasis reported by Mbow et al [4] are mediated mainly by IL-17F and IL-22 cytokines but not by IL-17A.

In contrast to DCs that promoted IL-17F and IL-22 secretion, *S. mansoni* egg-treated basophils did not modulate



**Figure 1.** Dendritic cells (DCs), and not basophils, mediate T-helper 17 responses to *Schistosoma mansoni*. Buffy bags from the healthy donors were obtained from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, after ethical committee permission was obtained. Peripheral blood mononuclear cells were isolated using Percoll density gradient centrifugation. Peripheral blood monocytes were isolated from peripheral blood mononuclear cells by using CD14 microbeads (Miltenyi Biotec), and DCs were derived by culturing monocytes in the presence of interleukin 4 (500 IU/10<sup>6</sup> cells) and granulocyte-macrophage colony-stimulating factor (1000 IU/10<sup>6</sup> cells; both from Miltenyi Biotec). Basophils were isolated from PBMCs with a basophil isolation kit II (Miltenyi Biotec). CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured (0.1 × 10<sup>6</sup> cells/200 μL/well) in X-vivo medium (Lonza) containing 10% human AB serum, either with interleukin 2 (100 IU/mL) and *S. mansoni* egg (500 eggs/10<sup>6</sup> cells) or with untreated DCs or with DCs treated with *S. mansoni* egg (500 eggs/10<sup>6</sup> DCs). In parallel, CD4<sup>+</sup>CD25<sup>-</sup> T cells were also cocultured with untreated basophils or basophils treated with *S. mansoni* eggs (500 eggs/10<sup>6</sup> basophils) in the presence of interleukin 3 (100 ng/0.1 × 10<sup>6</sup> basophils). The ratio of T cells to DCs or basophils was maintained at 5:1. After 4 days of coculture, the cells were harvested, and cell-free culture supernatants were collected. *A, B*, Harvested cells were restimulated with phorbol 12-myristate 13-acetate/ionomycin (Sigma) for 6 hours, with GolgiStop (protein transport inhibitor; BD Biosciences) during the last 3 hours. T-helper 17 responses were analyzed by flow cytometry with surface staining of CD4 (BD Biosciences) and intracellular staining of interleukin 17A (IL-17A; eBioscience). Representative dot plots show the proportion of IL-17A<sup>+</sup> T cells (*A*), and the percentages (mean ± standard error of the mean) of CD4<sup>+</sup>CD25<sup>-</sup> T cells positive for IL-17A are presented for different experimental conditions (*B*); data are from 5 independent experiments. *C–E*, Amounts of secretion for IL-17A (*C*), interleukin 17F (IL-17F; *D*), and interleukin 22 (IL-22; *E*) in the culture supernatants for the listed experimental conditions, as measured by enzyme-linked immunosorbent assay (ELISA; DuoSet ELISA kit [R&D Systems] for IL-17A and ELISA Ready-SET-Go kit [eBioscience] for IL-17F and IL-22). Data represent means ± standard errors of the mean from 5 independent experiments. Statistical significance was determined with 2-way unpaired Student *t* test. \**P* < .05; NS, not significant.

the production of IL-17A, IL-17F, and IL-22 (Figure 1*C, 1D*, and 1*E*). Altogether, these results suggest that basophils might be implicated in providing protective immunity during helminth infection by promoting the T-helper 2 microenvironment [9, 10]; however, Th17 cytokines production and associated pathogenesis in schistosomiasis are mediated mainly by DCs and not basophils.

## Notes

**Financial support.** This work was supported by the Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, Université Paris Descartes, Centre National de la Recherche Scientifique, and Institut Pasteur of Lille.

**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant

to the content of the manuscript have been disclosed.

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Received 20 September 2013; accepted 3 December 2013.  
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### The Journal of Infectious Diseases

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DOI: 10.1093/infdis/jiu025

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Disclosure of potential conflict of interest: P. Vadas has provided expert testimony in medical legal cases of injury due to anaphylaxis and owns US patent no. 8257697 ("Use of platelet activating factor acetylhydrolase as a biomarker for anaphylaxis"); however, no money was issued to him or his institution. G. Liss declares that he has no relevant conflicts of interest.

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Available online October 30, 2013.  
<http://dx.doi.org/10.1016/j.jaci.2013.09.012>

## Reply

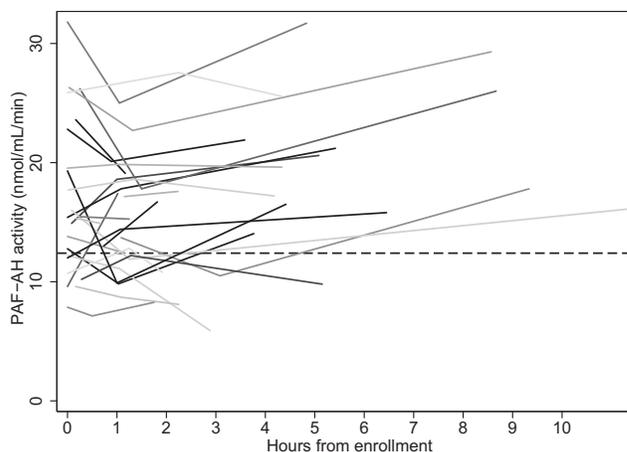
To the Editor:

Vadas and Liss<sup>1</sup> have restated their previous finding<sup>2</sup> that severe anaphylaxis is associated with low activity of the enzyme that deactivates the platelet-activating factor (PAF), PAF-acetylhydrolase (PAF-AH). They conclude that low PAF-AH activity should be used to identify at-risk individuals for targeted interventions. This assumes that low PAF-AH activity will identify those most likely to benefit. However, most people experiencing severe anaphylaxis do not have low PAF-AH activity,<sup>3</sup> yet may equally benefit from the same interventions. Another assumption is that low PAF-AH activity is a driver of severity rather than a consequence of it. Both Vadas et al<sup>1,2</sup> and our group<sup>3</sup> used samples collected during anaphylaxis, which may alter PAF-AH activity through oxidative stress or consumption/deactivation of the enzyme by the PAF. It is possible that PAF-AH activity might then return to normal in many cases. Bansal et al<sup>4</sup> highlighted this potential problem and found no difference in PAF-AH activity between 40 controls and 59 people who had previously experienced severe anaphylaxis.

In our article, we reported PAF-AH activity from the first sample taken on arrival in the emergency department, because this was the only one that was reliably collected across all sites.<sup>3</sup> However, in 26 cases with severe anaphylaxis, we were also able to measure PAF-AH activity in blood samples taken 1 hour after arrival and at discharge. There was substantial variability over time, with some cases showing a normalization of PAF-AH activity by the time of discharge (Fig 1). Unfortunately we did not obtain convalescent samples for comparison.

There are also potential problems with assay variability and standardization across sites. Vadas et al<sup>2</sup> used a cutoff value of 20 nmol/mL/min or less, we<sup>3</sup> used a cutoff of 12.4 nmol/mL/min or less to define low PAF-AH activity on the basis of the first centile of a panel of 30 healthy controls, and Bansal et al<sup>4</sup> found even lower PAF-AH activity in healthy controls (mean  $9 \pm 5$  nmol/mL/min).

Before we entertain the use of PAF-AH activity to target people for interventions to prevent anaphylaxis, we first need to improve assay standardization and confirm that convalescent measurements taken weeks to months after a reaction still correlate with reaction severity. We then need to determine



**FIG 1.** Changes in PAF-AH activity over time in 26 patients presenting to the emergency department with severe anaphylaxis. The lower limit of healthy normal range for our laboratory (12.4 nmol/mL/min) is represented by the dashed horizontal line.

whether interventions based on this result can indeed influence outcomes.

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Disclosure of potential conflict of interest: S. Brown has received grants from the National Health and Medical Research Council, the Royal Perth Hospital Medical Research Foundation, the Fremantle Hospital Medical Research Foundation, and the Food Allergy and Anaphylaxis Network. The rest of the authors declare that they have no relevant conflicts of interest.

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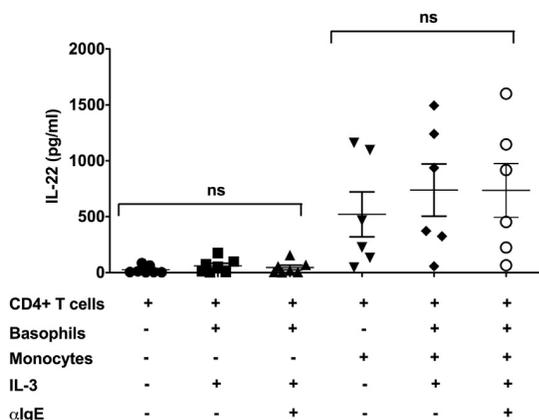
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Available online October 30, 2013.  
<http://dx.doi.org/10.1016/j.jaci.2013.09.013>

## Human basophils lack the capacity to drive memory CD4<sup>+</sup> T cells toward the IL-22 response

To the Editor:

Gaudenzio et al<sup>1</sup> reported that human mast cells drive memory CD4<sup>+</sup> T cells toward IL-22 producers. They found that mast cells form synaptic-like contacts with CD4<sup>+</sup> T cells and promote



**FIG 1.** Human basophils lack the capacity to drive memory CD4<sup>+</sup> T cells toward the IL-22 response. Secretion of IL-22 (in picograms per milliliters,  $n = 6-7$ ) by memory T cells that were cultured alone or with various combinations of activated basophils and peptidoglycan-stimulated monocytes is shown. Statistical analysis was done by using 1-way ANOVA, and comparison between sets of results was assessed by using the Tukey post-test. *ns*, Not significant.

IL-22-producing CD4<sup>+</sup> T cells through a TNF- $\alpha$ /IL-6-dependent mechanism. Thus these results point toward a role for mast cells in driving T<sub>H</sub> responses in inflammatory conditions.

Tissue-resident mast cells share several common features with basophils.<sup>2,3</sup> Both types of cells release various cytokines on activation, including IL-4, IL-6, and IL-13; express common receptors, such as Fc $\epsilon$ RI, CD200R3, C3aR, C5aR, IL-3 receptor, IL-18 receptor, and IL-33 receptor; and release several inflammatory mediators, such as histamine and leukotrienes. In addition, basophils are also known to recruit to the inflamed tissues. In view of these common features, similar activation mechanisms, and cytokine profiles, we explored whether human basophils, similar to mast cells, possess the capacity to promote IL-22 responses from CD4<sup>+</sup> T cells.

First, we examined the direct effect of basophils on IL-22 responses from CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cells. Basophils were activated either by IL-3 alone or through IL-3 and Fc $\epsilon$ RI cross-linking (see the **Methods** section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). We found that neither IL-3-primed nor Fc $\epsilon$ RI-activated basophils alone could promote IL-22 from memory CD4<sup>+</sup> T cells (Fig 1). These results imply that unlike mast cells, basophils are poor inducers of IL-22 responses from CD4<sup>+</sup> T cells.

Activation and expansion of CD4<sup>+</sup> T cells implicate coordination of 4 different signals, including activation of professional antigen-presenting cells (APCs) through pattern-recognition receptors (signal zero), interactions of antigen-loaded HLA-DR with T-cell receptor-CD3 complexes (signal 1) and costimulatory molecules (signal 2), and signaling events mediated by T cell-polarizing cytokines (signal 3). Several reports, including ours, have recently demonstrated that circulating human basophils lack the features of APCs to mediate T-cell responses. This inability of basophils to promote T-cell responses was mainly due to the absence of HLA-DR and the costimulatory molecules CD80 and CD86. Thus the lack of T-cell receptor- and costimulatory molecule-mediated signals might explain the inability of human basophils to mediate IL-22 responses.<sup>4,5</sup>

However, the above results did not provide clues on the indirect effect of basophils in promoting APC-mediated IL-22 responses.

Therefore by using Toll-like receptor (TLR)-activated monocytes, we determined whether circulating human basophils are capable of enhancing IL-22 from CD4<sup>+</sup> T cells. Monocytes were pulsed with the TLR2 agonist peptidoglycan and subsequently cocultured with memory CD4<sup>+</sup> T cells either in the presence or absence of activated basophils. We found that TLR-activated monocytes promoted IL-22 from memory CD4<sup>+</sup> T cells. However, IL-3-treated basophils did not further amplify monocyte-mediated IL-22 responses (Fig 1). Similar results were also obtained in the presence of Fc $\epsilon$ RI-activated basophils. These results suggest that circulating human basophils lack the ability to augment APC-mediated IL-22 responses.

In summary, our data indicate that although basophils share several common properties with mast cells, unlike these cells, basophils do not possess the capacity to drive memory CD4<sup>+</sup> T cells toward IL-22 producers, either directly or through APCs.

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Supported by Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, Université Paris Descartes, European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement HEALTH-2010.2.4.5-2 ALLFUN (to J.B.).

Disclosure of potential conflict of interest: J. Bayry has received a grant from the European Community's Seventh Framework Programme. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online November 4, 2013.  
<http://dx.doi.org/10.1016/j.jaci.2013.09.024>

## Reply

To the Editor:

We thank Sharma et al<sup>1</sup> for their interest in our recently published article.<sup>2</sup> In our study we demonstrate that primary human mast cells can express MHC class II and costimulatory molecules upon treatment with IFN- $\gamma$  and can form functional immunologic synapses with previously activated T cells. We also show that cognate interaction between mast cells and freshly isolated memory CD4<sup>+</sup> T cells leads to the generation of T<sub>H</sub>22 and IL-22/IFN- $\gamma$ -producing T<sub>H</sub> cells.<sup>2</sup> In addition, we previously demonstrated that IFN- $\gamma$ -primed mouse mast cells express MHC class II and costimulatory molecules and form immunologic synapses with

## METHODS

### Isolation of circulating human basophils, monocytes, and memory T cells

Buffy bags from healthy donors were obtained from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethics committee permission was obtained for the use of these buffy bags (no. 12/EFS/079). Basophil-rich fractions of PBMCs were obtained by using Percoll density gradient centrifugation. Basophils from these basophil-rich PBMCs were isolated by using the Basophil Isolation Kit II (Miltenyi Biotec, Paris, France). Monocytes were isolated from PBMCs by using CD14 magnetic microbeads (Miltenyi Biotec). For isolation of memory T cells, untouched total CD4<sup>+</sup> T cells were purified by means of negative selection with the CD4<sup>+</sup> T Cell Isolation Kit II (Miltenyi Biotec). Furthermore, untagged memory (CD45RO<sup>+</sup>) T cells were subsequently isolated from total CD4<sup>+</sup> T cells by positively selecting naive (CD45RA<sup>+</sup>) T cells with CD45RA microbeads (Miltenyi Biotec). Finally, CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cells were obtained by depleting CD25<sup>+</sup> cells with CD25 microbeads (Miltenyi Biotec). The purity of various cellular populations was greater than 95%.

### Coculture of basophils and monocytes with CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cells

CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cells were cultured in U-bottom, 96-well plates (0.1 × 10<sup>6</sup> cells/200 μL per well) in X-vivo-10% AB

serum and IL-2 (100 IU/mL) either alone or with IL-3 (100 ng/10<sup>6</sup> cells)-primed basophils, with IL-3 and anti-IgE (10 ng/0.1 million cells)-treated basophils, with monocytes stimulated with peptidoglycan (5 μg/0.5 million cells), with peptidoglycan-stimulated monocytes and IL-3-primed basophils, or with peptidoglycan-stimulated monocytes along with IL-3 and anti-IgE-treated basophils. The ratio of T cells and monocytes or basophils was maintained at 5:1. After 4 to 5 days of culture, cell-free culture supernatants were collected for analysis of IL-22.

### IL-22 analysis

Levels of IL-22 (ELISA Ready-SET-Go; eBioscience, San Diego, Calif) in cell-free culture supernatants were quantified by means of ELISA. The detection limit was 8 pg/mL.

### Statistical analysis

The significance of differences was assessed by using 1-way ANOVA, and comparison between sets of results was assessed by using the Tukey post-test. *P* values of less than .05 were considered statistically correlated. Prism 5 software (GraphPad Software, La Jolla, Calif) was used for statistical analyses.

**The role of basophils in autoimmune and inflammatory diseases is independent of human Th17 expansion**

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**Abbreviated Title:** basophils and human Th17 response

**Word Count:** Text: 2310, Abstract: 127

## **Abstract**

Basophils are the rare granulocytes and play an important role in the polarization of Th2 responses and protection against helminth parasites. Recent reports indicate that basophils have a role in the pathogenesis of several inflammatory and autoimmune diseases. Importantly, Th17 cells are also implicated in the pathogenesis of some of these diseases including asthma, systemic sclerosis, chronic urticaria, lupus, Henoch-Schönlein purpura and bullous pemphigoid. Therefore, we explored whether basophils contribute to pathogenesis of inflammation by promoting the expansion of human Th17 cells. We show that basophils lack the capacity to expand Th17 cells and to induce their cytokines either directly or indirectly via professional antigen presenting cells. These data thus indicate that the role of basophils in autoimmune and inflammatory diseases is independent of human Th17 expansion.

**Key words:** autoimmune diseases; inflammation; basophils; IL-17; Th17

## **Introduction**

Basophils are the rare granulocytes and represent less than 1% of circulating leukocytes. They play an important role in the polarization of Th2 responses and in the protection against helminth parasites [1, 2]. Recent studies have identified several surface markers of human and mouse basophils that could be used for the identification and isolation of these cells. These markers include CD49b (DX5), CD123 (IL-3 receptor  $\alpha$  chain), CD200R3 (a disulfide-linked dimeric CD200R-like receptor belonging to the immunoglobulin superfamily), CD203c, 2B4 (or CD244, a 66-kDa protein from the CD2 family), CCR2, CCR3, CD45R (intermediate level of expression) and Fc $\epsilon$ RI<sup>+</sup>. Further, in contrary to mast cells, basophils are c-Kit<sup>-</sup> (CD117) and could be used to discriminate basophils from mast cells in the tissues [2].

Since long time, basophils have been neglected in immunology due to their low number in the circulation and their shared features with tissue-resident mast cells. However, recent studies indicate that basophils have a major impact on the immune responses and diverse roles of these cells in autoimmune and inflammatory diseases are emerging. Because basophils express several sensing molecules including Fc $\epsilon$ RI, toll-like receptors (TLRs such as TLR2 and TLR4) and receptors for various cytokines including IL-3, IL-33 and IL-25, basophils can readily respond to various stimuli and release immune modulators such as cytokines, chemokines, histamine and lipid mediators [2]. Therefore, a higher number of activated basophils could tilt the homeostatic balance of the immune system leading to inflammatory conditions.

Basophils orchestrate IgE-mediated inflammation in chronic allergic dermatitis. In asthma, basophils act as accessory cells to provide Th2 environment and enhance dendritic cell-mediated Th2 responses. It is suggested that basophils contribute to the

pathogenesis of asthma during effector phase [3]. Recent reports also indicate that basophils have a role in the pathogenesis of autoimmune diseases such as systemic lupus erythematosus (SLE) and lupus nephritis. The pathogenic role of basophils in these disorders are mainly due to the function of basophils in the polarization of Th2 responses by providing IL-4, IL-13 and thymic stromal lymphopoietin (TSLP) and mediating activation and differentiation of B cells and enhancing humoral responses via BAFF [1, 4]. By using mice that lack Src family protein tyrosine kinase Lyn (Lyn<sup>-/-</sup> mice) and patients with SLE, Rivera and colleagues demonstrate that basophils can promote lupus nephritis [5]. Thus, they report that in Lyn<sup>-/-</sup> mice, autoreactive IgE mediate activation of basophils and homing them to lymph nodes. In lymph nodes, these basophils promote Th2 differentiation and enhance the production of auto-reactive antibodies that cause lupus-like nephritis. Interestingly, SLE patients also had elevated dsDNA-specific serum IgE and presence of activated basophils that express CD62L in the lymph nodes and spleen.

Plasma levels of histamine are increased in systemic sclerosis patients and that basophils appear to be implicated in the pathogenesis of systemic sclerosis. In fact, basophils from systemic sclerosis patients had shown an increased ability to release histamine [6]. It was found that inhibitory effect of sodium on basophil histamine release was impaired in systemic sclerosis patients [7].

By using a murine model, a recent report shows that TSLP-elicited basophil responses promote eosinophilic esophagitis, an inflammatory disease associated with food allergy [8]. The inflammation in this model was independent of IgE. Further, increased basophil responses were also detected in the esophageal biopsy tissue of patients with eosinophilic esophagitis and authors reported a genetic link between a

gain-of-function polymorphism in TSLP and amplified responses of peripheral basophils [8].

Basophils have an ability to migrate to mucosal and skin surfaces and are detected in the skin lesions of various inflammatory and autoimmune skin diseases such as atopic dermatitis, prurigo, urticaria, bullous pemphigoid, eosinophilic pustular folliculitis, Henoch-Schönlein purpura and dermatomyositis [9]. Basophils express CCR3 and in response to Th2-type cytokines IL-4 and IL-13 dermal fibroblasts produce CCL11 and CCL26, the ligands for CCR3. The recruitment of basophils to site of lesion would further boost Th2 environment. Ito et al., report that urticarial lesions, bullous pemphigoid and eosinophilic pustular folliculitis had high basophil densities, whereas concentration of basophils in prurigo and Henoch-Schönlein purpura was moderate and atopic dermatitis showed lowest tissue basophil densities. Also, circulating basophils from patients with inflammatory skin diseases such as urticarial displayed an activated phenotype as assessed by the basal level expression of CD203c, IL-3 hyper-responsiveness and enhanced histamine release upon surface IgE cross-linking [9, 10]. The reasons for activated phenotype of basophils in urticarial patients are not completely known. However, stimulating factor(s) in the sera could be one of the reasons as sera from patients with chronic idiopathic urticaria increased the expression of CD203c on basophils [11]. Of note, depletion of IgG in the representative sera from chronic idiopathic urticaria patients resulted in significant decrease in CD203c expression on donor basophils. These results are consistent with the fact that about 30-40% patients with chronic idiopathic urticaria (autoimmune urticaria) show the presence of a functional IgG antibody to the  $\alpha$  subunit of the Fc $\epsilon$ RI or to IgE which trigger activation of basophils.

The aforementioned autoimmune and inflammatory diseases are characterized by the huge infiltration of CD4<sup>+</sup> T cells and in particular Th2 cells. However, a newly identified subset of T cells namely Th17 cells also reported to be implicated in the pathogenesis of many of the above diseases [12-15]. Th17 cells express lineage specific transcription factor RORC and IL-17A is the prototype cytokine of these cells. In addition, Th17 cells secrete other inflammatory mediators such as IL-17F, IL-21, IL-22, GM-CSF and CCL20 [12]. Importantly, differentiation of regulatory T cells (Tregs) and Th17 cells from naïve CD4<sup>+</sup> T cells is reciprocally regulated due to critical role played by the TGF- $\beta$  to induce both FoxP3 and RORC [16]. Thus, increased activation of Th17 cells in autoimmune and inflammatory diseases where basophils are one of the players of pathogenesis of these pathologies raises the possibility that basophils might support Th17 response. As basophils have an important role in the regulation of immune responses such as T and B cell responses, we explored whether basophils contribute to pathogenesis of inflammation by promoting the expansion of human Th17 cells.

## **Materials and methods**

### ***Isolation of circulating human basophils and monocytes***

Buffy coats of healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France upon ethical committee permission (N°12/EFS/079). Basophils from buffy coats were isolated by two-step process. By percoll density gradient centrifugation, we first obtained basophil-rich fraction of peripheral blood mononuclear cells (PBMC). This basophil-rich fraction of PBMC was subjected to MicroBead-based negative isolation of basophils by using basophil isolation kit II (Miltenyi Biotec, Paris, France) [17]. Monocytes from PBMC were purified by using CD14 Microbeads (Miltenyi Biotec).

### ***Isolation of memory CD4<sup>+</sup> T cells***

To isolate memory CD4<sup>+</sup> T cells, untouched total CD4<sup>+</sup> T cells were first purified from PBMC by using CD4<sup>+</sup> T-cell isolation kit II (Miltenyi Biotec). Further, by using CD45RA MicroBeads (Miltenyi Biotec), naïve CD4<sup>+</sup>CD45RA<sup>+</sup> T cells were depleted from total CD4<sup>+</sup> T cells. Finally, CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cells were obtained by depleting CD25<sup>+</sup> cells with CD25 MicroBeads (Miltenyi Biotec).

### ***Co-culture of basophils and monocytes with CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cells***

Memory CD4<sup>+</sup> T cells were plated in U-bottomed 96 wells plate (0.1x10<sup>6</sup> cells/200 µl/well) in X-vivo-10% human AB serum and IL-2 (100 IU/ml) either alone; or with basophils that were primed with IL-3 (100 ng/10<sup>6</sup> cells) or IL-3 and anti-IgE (10 ng/0.1x10<sup>6</sup> cells); or with peptidoglycan-stimulated monocytes (5 µg / 0.5 x10<sup>6</sup> cells); or with peptidoglycan-stimulated monocytes and IL-3-primed basophils; or with peptidoglycan-stimulated monocytes and IL-3-anti-IgE-treated basophils. The ratio of memory CD4<sup>+</sup> T cells and monocytes and/or basophils maintained at 5:1. After four to

five days of culture, the cells were harvested and cell-free culture supernatants were collected for the analysis of IL-17A and IL-17F. The cells were processed for staining and flow cytometry as described below.

#### ***Intracellular staining and flow cytometry***

The harvested cells were re-stimulated with phorbol 12-myristate 13-acetate/ionomycin (Sigma, Saint Quentin Fallavier, France) for 6 hours, with GolgiStop (BD Biosciences, Le Pont de Claix, France) during last 3 hours. Surface staining was done with fluorescence-conjugated CD4 mAb (BD Biosciences) and fixable viability dye (eBioscience, Paris, France), in order to gate and analyze viable CD4<sup>+</sup> T cells. Further, cells were fixed, permeabilized (Fix/Perm; eBioscience), and incubated at 4°C with fluorescence-conjugated mAbs to IFN- $\gamma$  (BD Biosciences) and IL-17A (eBioscience). The stained cells were subjected to flow cytometry (BD LSR II). Ten thousand cells were acquired for each sample and data were processed by using FACS DIVA software (BD Biosciences).

#### ***Statistical analysis***

Statistical analysis was done by one-Way ANOVA (Friedman test) using Prism 5 software (GraphPad softwares). Values of P<0.05 were considered as statistically correlated.

#### ***Cytokines analysis***

Levels of IL-17A (DuoSet ELISA kits, R&D Systems) and IL-17F (ELISA Ready-SET-Go, eBioscience) in cell-free culture supernatants were quantified by ELISA. The detection limits were 15 pg/mL for IL-17A and 30 pg/mL for IL-17F.

## Results and Discussion

First, we explored the direct effect of basophils on the expansion of Th17 cells. As stimulated basophils are known to secrete variety of cytokines and other chemical mediators, we also examined if enhanced degranulation of basophils through FcεRI cross-linking would augment Th17 responses. To analyze this, IL-3-primed basophils were co-cultured with CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells either in the presence or absence of FcεRI cross-linking. To avoid nonspecific stimulatory effects of xeno-proteins in the fetal calf serum, we utilized X-vivo medium-containing 10% human AB serum for the experiments. We observed that neither IL-3-primed nor FcεRI-activated basophils alone could amplify IL-17<sup>+</sup> Th17 cells from memory T cells subsets (Fig. 1A and 1B). The percentage of IL17<sup>+</sup>/IFN-γ<sup>-</sup> and IL-17<sup>+</sup>/IFN-γ<sup>+</sup> T cells remained unaltered in the presence of either IL-3-primed or FcεRI-activated basophils. In addition, basophils did not activate Th17 cells to secrete Th-17-derived cytokines. Only marginal changes in the secretion pattern of IL-17A and IL-17F were observed (Fig. 2A and 2B). This observation implies that basophils alone are poor inducers of Th17 cell expansion and hence ruled out the possibility of the direct association of basophils in the development of Th17 responses.

Various receptor-ligand interactions between antigen presenting cells (APC) and responder CD4<sup>+</sup> T cells, and cytokine milieu in the microenvironment determine the activation, polarization and expansion of CD4<sup>+</sup> T cells. Previous reports showed that murine basophils at secondary lymphoid organs display features of professional APC and promote Th2 responses [18-20]. However, these reports are contradictory due to the basophil depletion method employed [21, 22]. In contrast to murine basophils, several reports including ours demonstrated that circulating human basophils lack

HLA-DR and co-stimulatory molecules CD80 and CD86 and were unable to function as APC to promote T cell polarization [17, 23-25]. Although, stimulation of basophils with GM-CSF and IFN- $\gamma$  was shown to induce HLA-DR expression to a smaller extent in some donors, cells remained negative for co-stimulatory molecules [26]. Thus, the inability of human basophils to confer TCR- and co-stimulatory molecule-mediated signals to CD4<sup>+</sup> T cells might explain the lack of Th17 responses when CD4<sup>+</sup> T cells were co-cultured with basophils.

It is known that basophils secrete various inflammatory mediators and hence could influence the activation of other immune cells [2, 27]. Therefore, by mimicking closely the tissue microenvironment i.e. in the presence of activated APC that would provide all different signals required for CD4<sup>+</sup> T cell activation, we examined if circulating human basophils have an ability to promote Th17 cell expansion mediated by APC. Monocytes that were stimulated with TLR2-agonist peptidoglycan were utilized as APC. These activated monocytes were co-cultured with CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells either in presence of absence of basophils.

We found that IL-17<sup>+</sup> Th17 cells were significantly enhanced when memory CD4<sup>+</sup> T cells were co-cultured with monocytes, thus confirming the ability of activated APC to expand Th17 cells. Whereas, IL-3 treated basophils did not further amplify monocyte-mediated Th17 responses (Fig. 1A and 1B). Also, the proportion of IL17<sup>+</sup>/IFN- $\gamma$ <sup>-</sup> and IL-17<sup>+</sup>/IFN- $\gamma$ <sup>+</sup> T cells was not significantly altered in the presence of IL-3-primed basophils with APC (Fig. 1A and 1B). Interestingly, similar results were also obtained in the presence of Fc $\epsilon$ RI-activated-basophils. These flow-cytometry results were further confirmed by the analysis of secretion of Th-17-derived cytokines. Monocytes significantly enhanced the production of IL-17A and IL-17F by ten to fifteen times (Fig. 2A and 2B). Although there was a slight increase

in the production of these cytokines in the presence of basophils, the values were not statistically significant (Fig. 2A and 2B). Taken together, these results thus provide a pointer that circulating human basophils lack the capacity to enhance APC-mediated Th17 responses. Basophils have been shown to secrete small amounts of IL-6 that could explain marginal increase in the level of Th17 cytokines. As innate cells such as dendritic cells (DCs) and monocyte/macrophages are known to migrate to the inflammatory sites and could secrete massive quantities of Th17-amplifying cytokines, the basophil-secreted IL-6 effect would be nullified.

Recently Wakahara et al., shown that human basophils enhance Th17 responses upon interaction with memory cells [28]. The reasons for the discrepancies in the results are not clear. Differences in the serum source and stimulatory conditions could be the possible reasons. Based on their results and presence of basophils in the inflamed mucosal tissues, Wakahara et al., also suggested a role for basophils in the pathogenesis of inflammatory bowel disease [28]. However, on the contrary, a recent report using the colitis model demonstrates that basophils limit the disease activity in experimental murine colitis [29]. Also, due to failure of anti-IL-17A monoclonal antibody, the pathogenic role of Th17 cells in inflammatory bowel disease remains controversial [30].

Together, our results indicate that basophils lack the ability to augment Th17 cell responses either directly or via APC. Therefore, increased activation and accumulation of Th17 cells in various inflammatory diseases implicate the repercussion of stimulation of these cells by innate cells other than basophils such as either monocytes or DCs. Thus, basophil and Th17 cell responses in inflammatory diseases represent two independent but parallel events.

## **Acknowledgments**

Supported by European Community's Seventh Framework Programme [FP7/2007-2013] under Grant Agreement No: 260338 ALLFUN. We also thank the supports from Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie and Université Paris Descartes.

**Conflict of interests:** The authors declare no competing financial interests.

## **Author contributions**

M.S. performed experiments, analyzed the data and drawn the figures.

S.V.K. analyzed the data.

J.B. analyzed the data, drawn the figures and wrote the paper.

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## Figure Legends

**Fig 1.** Human basophils are mute-spectators in Th17 expansion. (A) Flow-cytometry analysis of intracellular IL-17 and IFN- $\gamma$  in the memory T cells that were cultured alone (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or combination of IL-3 and anti-IgE. (B) Percentage (mean $\pm$ SEM) of CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells positive for IL-17A<sup>+</sup> (n= 5) in the above experimental conditions. \*, P<0.05; ns, not-significant.

**Fig 2.** Human basophils do not promote Th17 cytokine secretion. The amount of secretion (pg/ml) of (A) IL-17A and (B) IL-17F in the culture supernatants of memory T cells that were cultured alone (T) or with basophils (T+B) or peptidoglycan-stimulated monocytes (T+M) or peptidoglycan-stimulated monocytes and basophils (T+M+B). Basophils were stimulated either with IL-3 or combination of IL-3 and anti-IgE. The cytokines were measured by ELISA. The data represent mean $\pm$ SEM from five independent experiments using cells from different donors. \*, P<0.05; ns, not-significant.

Figure 1:

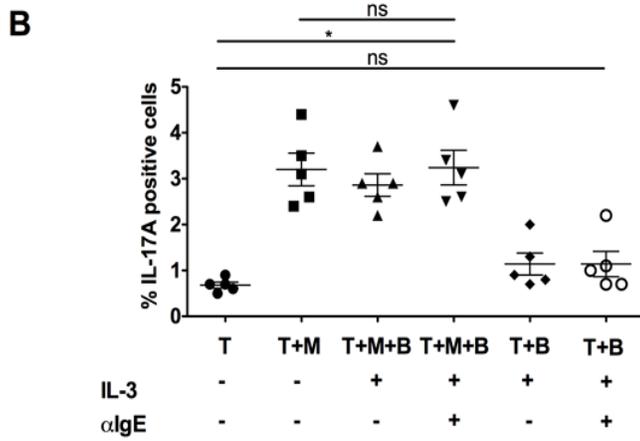
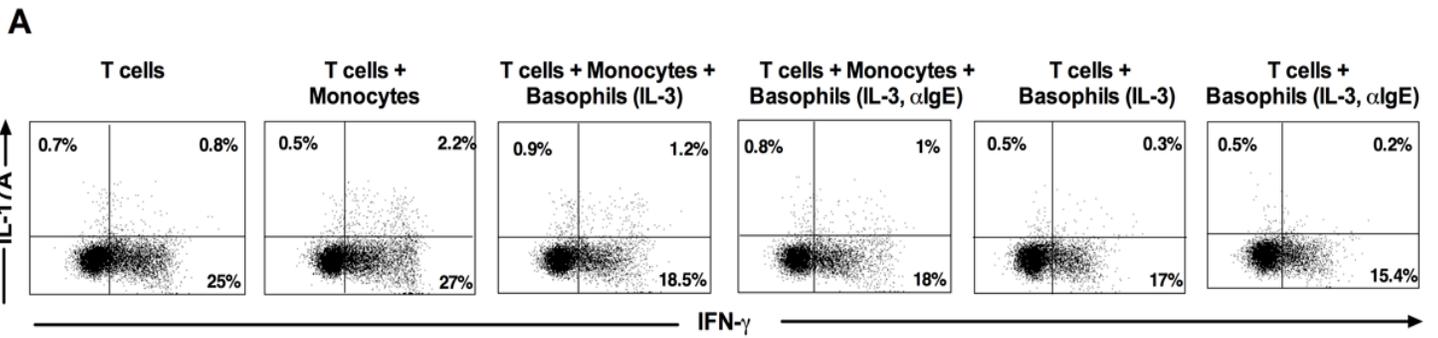
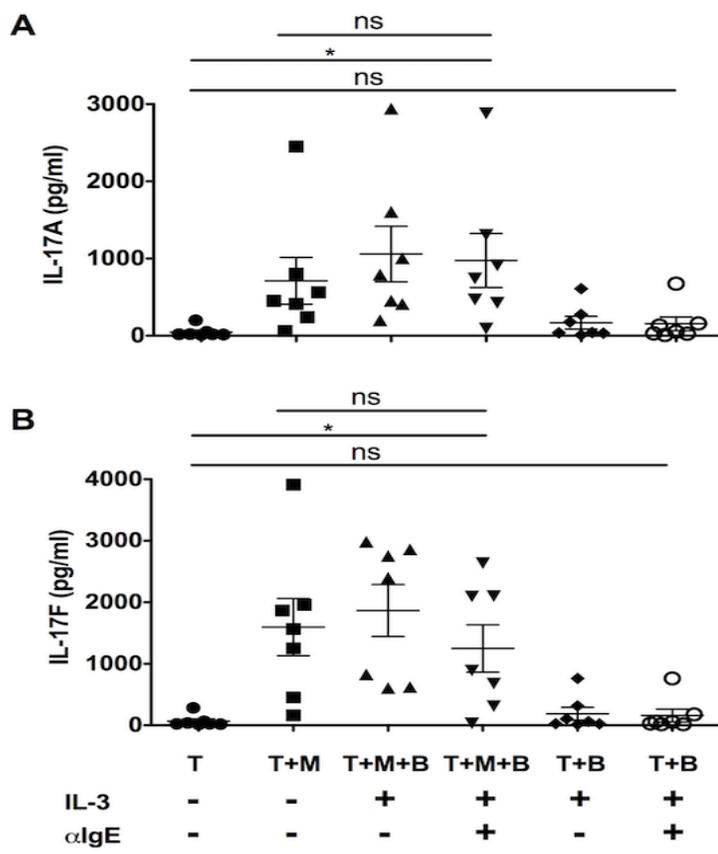


Figure 2:



## **Article 5- Résumés en français**

### **Titre: Les basophiles sont réfractaires à la régulation par les lymphocytes T régulateurs CD4<sup>+</sup>CD25<sup>+</sup>Foxp<sup>3</sup>**

Les basophiles, des cellules de l'immunité innée, jouent un rôle important dans le développement des réponses allergiques et inflammatoires. Puisque le maintien de l'homéostasie immune dépend des lymphocytes T régulateurs CD4<sup>+</sup>CD25<sup>+</sup>FoxP3, nous avons exploré l'interaction des basophiles avec les Tregs et la répercussion de cette dernière sur les fonctions des basophiles. De façon surprenante, les Tregs n'inhibent pas l'activation des basophiles. En effet, l'expression des marqueurs d'activation et le relargage de l'histamine par les basophiles ne sont pas affectés par les Tregs. Nous avons trouvé que la résistance au Tregs est due, au moins en partie, à l'absence de l'expression de CD80/CD86, HLA-DR et le récepteur de TGF-β. Ces derniers sont importants pour la reconnaissance par les molécules suppressives des Tregs, comme CTLA-4, LAG-3 et TGF-β. De plus, bien qu'une proportion considérable des basophiles expriment le récepteur d'IL10, l'activation des basophiles n'est pas modulée ou régulée par l'IL10.

Ainsi, nos résultats montrent que les basophiles, contrairement à la majorité des cellules immunes susceptible à l'effet suppresseur des Tregs, sont réfractaires à la régulation par les Tregs.

# **Basophils are refractory to suppression by human CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells**

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## **Abstract**

Basophils are innate immune cells, known to play a major role in the promoting allergic and inflammatory responses. As CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) are critical for the maintenance of immune homeostasis, we sought to investigate the interaction of Tregs with human basophils and its repercussion on basophil functions. Surprisingly, we found that Tregs did not inhibit the basophil activation as analyzed by the expression of activation markers and histamine release. While identifying the reasons for basophil resistance to Treg-mediated suppression, we found that human basophils did not express CD80/CD86, HLA-DR and TGF- $\beta$  receptor, the ligands for Treg-associated suppressive molecules CTLA-4, LAG-3 and TGF- $\beta$ . Although substantial proportion of basophils expressed IL-10 receptor, IL-10 did not suppress the activation of basophils regardless of the expression of receptor. Altogether, these results indicated that unlike other immune cells that are susceptible to Treg-mediated suppression, basophils are refractory to regulatory mechanism of Tregs.

## **Introduction**

Basophils are rare circulating granulocytes and have vastly been investigated for their role in allergic responses<sup>1</sup>. Several evidences suggest that in addition to the secretion of the mediators of hypersensitive reactions such as histamine and leukotriene, basophils upon activation can also produce variety of cytokines like IL-4, IL-13, IL-6, TSLP and BAFF<sup>2 3 4</sup>. These reports also confirmed the immunomodulation of T and B cell responses by basophils and thereby pointed towards role of basophils in establishing the link with adaptive immunity<sup>5 6</sup>. Further, basophils are also found to be implicated in the development of Th2 –associated and B cell-mediated autoimmune and inflammatory diseases<sup>7 8</sup>. Therefore, considering the impact of dysregulated function of basophils on the outcome immune responses in various pathological conditions, it is essential to investigate the regulatory mechanisms by which basophil functions are kept in check.

In this regard, regulatory T cells (Tregs) have been widely studied for their role in immune tolerance and in the maintenance of immune homeostasis<sup>9</sup>. Tregs prevent autoimmune and inflammatory responses<sup>10 11</sup> by exerting direct suppressive effects on various immune cells including dendritic cells, T cells, macrophages, B cells, NK cells and mast cells<sup>12 13 14 15</sup>. These functions of Tregs are mediated both by cell-associated molecules such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and soluble mediators such as TGF- $\beta$ . However, despite their major role in the pathogenesis of various inflammatory conditions, the regulation of basophil functions by Tregs is not yet known. Therefore, we explored the interaction of Tregs with basophils and its repercussion on basophil functions.

## **Methods**

### **Isolation of basophils and Tregs**

Blood from healthy donors was obtained from Centre Necker-Cabanel (Etablissement Français du Sang, Paris, France) and ethical committee permission was obtained for the use. Peripheral blood mononuclear cells (PBMC) were obtained from blood that was processed by percoll density gradient centrifugation<sup>16</sup>. Basophils were isolated from these PBMC by Basophil isolation kit II (Miltenyi Biotec, Paris, France). The purity of basophils was 92±5%. For isolating Tregs (CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>+</sup>), untouched CD4<sup>+</sup> T cells were first purified (CD4<sup>+</sup> T cell isolation kit II; Miltenyi Biotec) followed by subsequent purification of untouched memory T cells (CD45RO<sup>+</sup>) by depleting naïve T cells (CD45RA microbeads; Miltenyi Biotec). Further, CD25<sup>+</sup> Tregs were positively selected from the untouched memory T cell fraction by using CD25 microbeads (Miltenyi Biotec). CD4<sup>+</sup>CD45RO<sup>+</sup>CD25<sup>-</sup> memory T cell fraction was used as conventional T cells (Tconv) for comparison with Tregs. The purity of T cell populations was 94±3%.

### **Co-culture of basophils with Tregs or Tconv**

Basophils (1x10<sup>4</sup> cells per well/200 µL) were cultured in 96 well U-bottomed plate either alone or with IL-3 (10 ng/ml) or co-cultured with Tregs or Tconv at 1:4 ratio in serum-free X-VIVO 15 medium (Lonza) for 24 hours. IL-3 (10 ng/ml) was added to all the co-culture conditions for survival of basophils. Tregs/Tconv in the co-culture conditions were stimulated with plate-bound anti-CD3 mAb (0.5 µg/mL) and anti-CD28 mAb (0.5 µg/mL) (R & D systems). Further, to check functional response of Tregs/Tconv-educated basophils, anti-IgE (10 ng/ml) was added during last one hour of the culture. Basophil

response was examined by analyzing the expression of activation-associated markers by flow cytometry. Further, cell-free culture supernatants were harvested to measure histamine release.

In order to analyze the effect of IL-10 treatment on basophil activation, basophils ( $1 \times 10^4$  per well/200  $\mu$ L) were cultured either alone in X-VIVO 15 medium or treated with increasing concentration of rh IL-10 (eBiosciences, France) from 100 pg/ml to 10 ng/ml, for 24 hours. To check the basophil responsiveness upon IL-10 treatment, anti IgE (10 ng/ml) was added during last one hour of the culture and expression of activation markers was analyzed by flow cytometry.

#### **Flow cytometry and ELISA**

The cell surface staining was performed by using fluorochrome-conjugated monoclonal antibodies for CD80, CD86, CD203c, CD13 and Annexin V (all from BD Biosciences, Le Pont de Claix, France), IL-10R, TGF- $\beta$ R (R&D systems) and CD123 and Fc $\epsilon$ RI (Miltenyi Biotec). PI was purchased from Sigma-Aldrich (Lyon, France). The cells were then processed for flow cytometry (LSR II, BD Biosciences) and data were analyzed by BD FACS DIVA program. Histamine was measured in culture supernatants using histamine ELISA kit (Immunotech, France).

#### **Treg suppression assay**

To analyze Treg-mediated suppression on proliferation of T cells, purified Tconv cells were first labeled with cell trace violet dye (Life technologies, France) at 37°C for 20 minutes and then washed two times with RPMI 1640 to remove excess dye. These

stained Tconv ( $2 \times 10^5$  cells/ well/ 100 $\mu$ l) were then cultured in 96 well U-bottomed plate, previously coated with anti-CD3 mAb (0.5  $\mu$ g/mL). These cells were cultured in serum-free X-VIVO 15 medium, either alone or with purified Tregs in ratio 1:1. Soluble anti-CD28 mAb (0.5  $\mu$ g/mL) was added for complete activation of T cells. After four days, proliferation of Tconv was analyzed based on dilution of dye measured by flow cytometry.

### **Statistical analysis**

One-way ANOVA (repeated measures with Tukey's multiple comparison test) and paired Student's t-test were used to determine the statistical significance of the data.  $P < 0.05$  was considered significant.

## Results and discussion

Considering immuno-regulatory and suppressive functions of Tregs, we first assessed responsiveness of Treg-educated basophils for anti-IgE stimulation. The activation of basophils was analyzed based on the expression of markers including CD203c, CD13, CD69 as well as degranulation-associated markers CD63 and CD107a. We found significant increase in the expression of all the activation markers upon treatment with anti-IgE. However, surprisingly, Tregs did not inhibit the activation of basophils and the expression of various activation-associated markers of basophils was on par with that of control basophils and Tconv-educated basophils (Figure 1A- E). These data thus indicate refractoriness of basophils to Treg-mediated suppression.

We then investigated the effect of Tregs on the release of histamine from basophils when they received signaling by anti-IgE. Corroborating the phenotype results, we could not detect any indication of Treg-mediated suppression on basophil histamine release (Figure 1F). We confirm that the inability of Tregs to suppress activation of basophils was not due to either low purity of cells or due to defects in their functions. In fact, 94 % purified Tregs expressed FoxP3 and these Tregs suppressed the proliferation of effector T cells in a functional assay (Figure 1G and H). Altogether, these results indicated that unlike other immune cells that are susceptible to Treg-mediated suppression, basophils are refractory to regulatory mechanism of Tregs.

We then aimed at exploring the reasons for resistance of basophils to Treg-mediated suppression. Suppression of target cells by Tregs is attributed to both contact-dependent and –independent mechanisms<sup>17 18 19</sup>. The data from mice and human suggested that

CTLA-4 and LAG-3 play a critical role in contact-dependent suppression of target cells by Tregs<sup>20 21</sup>. In addition, granzyme A and Fas-FasL interactions were also implicated in exerting Treg-mediated cytotoxic effects on target cells<sup>19 22 23</sup>. As the functions of CTLA-4 and LAG-3 are mediated via CD80/CD86 and HLA-DR respectively, we analyzed the expression of these molecules on basophils. However, we could not detect the expression of both CD80 and CD86 on circulating basophils even following activation. Similarly, HLA-DR was also absent on basophils (Figure 2 A)<sup>24 25</sup>. We then explored whether the lack of expression of HLA-DR and co-stimulatory molecules on basophils is restricted only to circulating population or is pertinent to basophils in the secondary lymphoid tissues of humans.

Further, to confirm if Tregs could induce cytotoxic effects on basophils, we measured the viability of basophils cultured with either Tregs or Tconv, by annexin V and propidium iodide staining. However, no significant differences were observed in the level of viability of control basophils and T cell-co-cultured basophils (Figure 2B).

Several studies have demonstrated that Tregs-mediated suppressive functions are also mediated by cytokines and in particular TGF- $\beta$  and IL-10<sup>19 26 27</sup>. To investigate effect of these mediators on basophils, we analyzed the expression of receptors for on basophils both the cytokines TGF- $\beta$  and IL-10. TGF- $\beta$  receptor (TGF- $\beta$ R) was not expressed by basophils (Figure 2C) and hence ruled out the effect of Treg-derived TGF- $\beta$  on basophils, a substantial proportion of basophils ( $36 \pm 5\%$ , n=5) expressed receptor for IL-10 on their surface (Figure 2C).

Despite the presence of IL-10 receptor on the surface, the unresponsiveness of basophils to Tregs could be due to either heterogeneity of basophils regarding IL-10 receptor

expression or insensitivity of basophils to IL-10. Tregs are reported to produce upto 8 pg/ml/2 × 10<sup>5</sup> of cell IL-10<sup>28</sup>. Therefore, basophils were treated with increasing concentrations of IL-10 (from 100 pg to 10 ng/ml) for 24 hours and stimulated with anti-IgE during last one hour of culture. The repercussion of IL-10 exposure on phenotype of basophils was compared among IL-10R<sup>+</sup> and IL-10R<sup>-</sup> subsets. We found that even at highest concentrations (10 ng), IL-10 did not modify the expression of activation markers on basophils regardless of IL-10R<sup>+</sup> or IL-10R<sup>-</sup> subsets (Figure 2D). Taken together, these results thus provide possible explanations for refractoriness of basophils to Treg-mediated suppression.

Tregs were demonstrated to inhibit FcεRI-induced degranulation of murine mast cells, the tissue-resident counterpart of basophils, in an OX40-dependent manner<sup>12 29</sup> whereas TGF-β/TGF-βR interaction was found to be necessary for enhancing IL-6 production from mast cells<sup>29</sup>. Tregs also inhibited FcεRI expression and its signaling in murine mast cells<sup>30</sup>. Although these results are not yet confirmed in humans, our results showed that Tregs did not affect degranulation process of human basophils as measured by histamine release and also did not inhibit FcεRI expression. Despite human basophils share several common features with mast cells, they do display distinct features. Unlike basophils, human mast cells express co-stimulatory and antigen-presenting molecules and induce activation and proliferation of CD4<sup>+</sup> T cells<sup>31 24 25</sup>. Based on present data, we suggest that as compared to mast cells, human basophils also display discrete features of regulation.

A recent report showed that chronic helminth infection in mice reduced basophil responsiveness in an IL-10-dependent manner and inhibited basophil responsiveness to

IgE cross-linking <sup>32</sup>. However, it is evident that mice and human basophils display distinct differences in their surface phenotype and functions. Thus, unlike murine, human basophils did not express antigen-presenting and co-stimulatory molecules. In addition, human basophils were also unresponsive to IL-10 and did not inhibit activation of basophils upon IgE-cross-linking. Alternatively, as IL-10 is known to have suppressive effects on other immune cells and that basophils receive activation-associated signals both from innate and adaptive immune cells, the observed IL-10-dependent suppression of murine basophil response could be indirect rather than its direct effect on basophils.

## Figure legends

**Figure 1: basophils are refractory to Treg-mediated suppression.** Basophils ( $1 \times 10^5$  per well/100  $\mu$ L) were cultured in 96 well U-bottomed plates either alone or with IL-3 (10 ng/ml) or co-cultured with Tregs or Tconv ( $3 \times 10^5$  per well/100  $\mu$ L) at 1:4 ratio in serum-free X-VIVO 15 medium for 24 hours. IL-3 (10/ml) was added to all the co-culture conditions for survival of basophils. Tregs/Tconv in the co-culture conditions were stimulated with plate-bound anti-CD3 mAb (0.5  $\mu$ g/mL) and soluble anti-CD28 mAb (0.5  $\mu$ g/mL). Further, to check functional response of Tregs/Tconv-educated basophils, anti-IgE (10 ng/ml) was added during last one hour of the culture. **(A-C)** Basophil response was determined by analyzing the expression of activation-associated markers CD203c, CD13 and CD69 and **(D and E)** degranulation-associated markers CD63 and CD107a; by flow cytometry. Percentage of cells expressing denoted markers and mean fluorescence intensity (MFI) values are indicated. **(F)** Effect of Tregs or Tconv cells on histamine release from basophils upon anti-IgE stimulation. Data (mean  $\pm$  SEM) are from six independent experiments. **(G)** Foxp3 expression on purified Tregs as analyzed by flow cytometry. **(H)** Suppression of effector T cell proliferation by purified Tregs. Tconv cells were labeled with cell trace violet dye and were cultured in serum-free X-VIVO 15 medium either alone (left panel) or with purified Tregs in ratio 1:1 (right panel). T cells were stimulated with plate-bound anti-CD3 mAb (0.5  $\mu$ g/mL) and soluble anti-CD28 mAb (0.5  $\mu$ g/mL). After four days, proliferation of Tconv cells was analyzed based on dilution of cell trace violet dye.

**Figure 2: Exploration of reasons for resistance of basophils to Treg-mediated suppression.** (A) Expression of co-stimulatory molecules CD80/CD86 and antigen presenting HLA-DR on basophils. (B) Viability of basophils under different experimental conditions. Basophils were cultured either alone or with IL-3 (10ng/ml) or with activated (plate bound anti-CD3 and soluble anti-CD28) Tregs or Tconv cells for 24 hours and anti-IgE (10 ng/ml) was added during last one hour of culture. Viability of basophils was analyzed by the surface expression of apoptotic maker, Annexin V and necrotic marker, propidium iodide (PI). Data (mean  $\pm$ SEM) are from three independent experiments. (C) Expression of TGF- $\beta$  and IL-10 receptors on basophils. Representative dot-plot of five independent donors. (D) Effect of IL-10 on the expression of basophil activation markers. Basophils ( $1 \times 10^4$  per well/200  $\mu$ L) were cultured either alone in X-VIVO 15 medium or treated with various concentration of rhIL-10 for 24 hours. Anti-IgE (10 ng/ml) was added during last one hour of the culture and expression (MFI) of activation markers CD203c, CD123, CD69, CD13 and Fc $\epsilon$ RI on basophils were analyzed on IL-10R<sup>+</sup> and IL-10R<sup>-</sup> subsets based on the gating strategy depicted in (C). Data (mean  $\pm$  SEM) are from four independent experiments.

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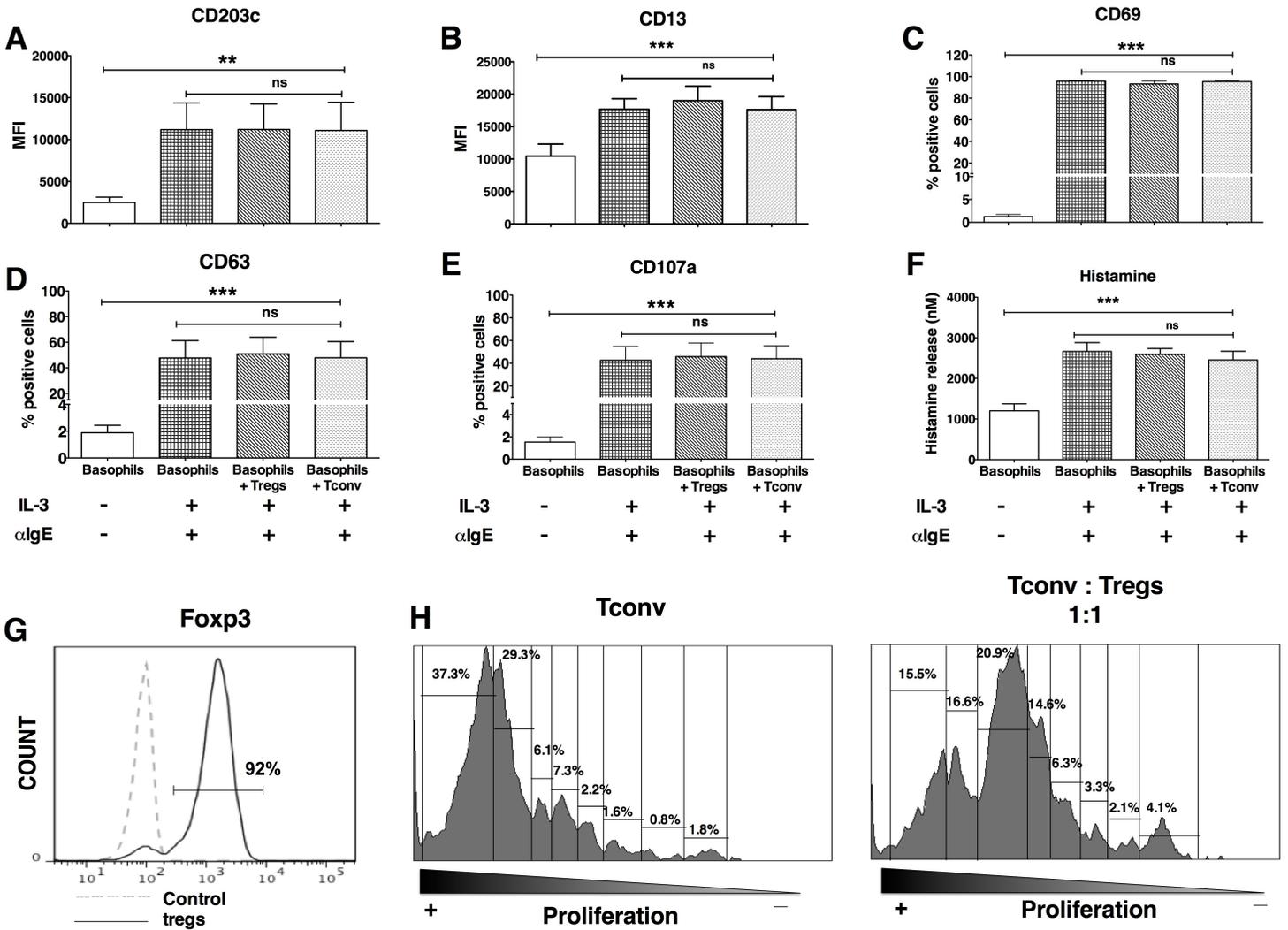
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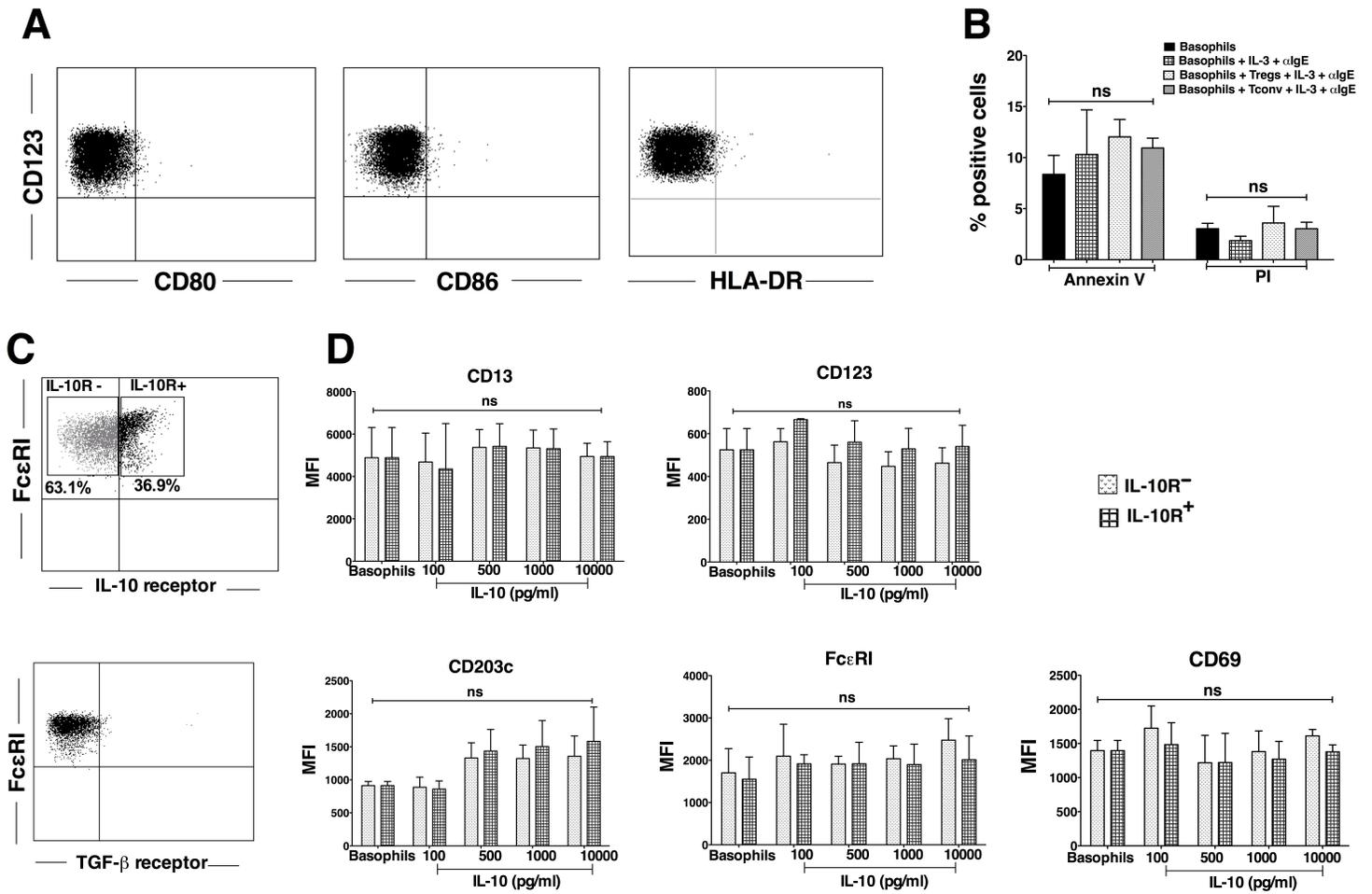
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**Figure 1**



# Figure 2



# Discussion

Basophils are least represented granulocytes in the circulation and are known for their role in allergic reactions as well as in providing protective immunity against helminth parasites. Various reports have demonstrated that basophils are the initial source of IL-4 and critical for driving T cell response towards Th2 phenotype. Cytokines such as IL-3, IL-33 and TSLP were identified as regulators of basophil production, survival and activation (Voehringer, 2012). Basophils could also respond to IgE and IgD-mediated signals and secrete variety of cytokines and chemical mediators upon activation (Chen et al., 2009) (Chirumbolo, 2012). Apart from secretion of vasoactive mediators such as histamine and leukotriene, basophil activation is also marked by release of IL-4, IL-13, IL-6 and TSLP (Chirumbolo, 2012). The implication of basophils in context to allergic and Th2-associated responses has vastly been studied. Recently reported studies also revealed newly identified role of basophils in the pathogenesis of inflammatory and autoimmune diseases such as lupus nephritis, urticaria and eosinophil esophagitis (Charles et al., 2010) (Noti et al., 2013) (Yasnowsky et al., 2006). However, despite several reports demonstrating the role of basophils in modulating T and B cell responses, crucial aspects of 'human' basophil function are still not well established.

In my thesis work, I addressed some key questions regarding cellular and molecular cross talk between human basophils and CD4<sup>+</sup> T cells.

- I investigated the role of human circulating basophils as APCs in driving Th1, Th2 and Th17 responses.
- In extension to exploration of cross-talk between basophils and CD4<sup>+</sup> T cells, I investigated role of CD4<sup>+</sup> CD25<sup>+</sup> Tregs in regulating basophil functions.

## **1. Basophils as APC in mediating Th1 and Th2 responses**

Newly discovered role of basophil as APCs has been demonstrated in various reports. Three independent reports in murine models showed that basophils could act as predominant APCs in inducing Th2 responses against helminth parasite, protein allergen and proteases (Perrigoue et al., 2009) (Sokol et al., 2009) (Yoshimoto et al., 2009). These studies demonstrated that basophils express antigen presenting MHC-II and co-stimulatory molecules CD80 and CD86. Thus, by presenting peptide antigen to CD4<sup>+</sup> T cells in the context of MHC-II and by producing large amounts of IL-4, basophils contributed to Th2/IgE responses. Further, lymph node recruitment of basophils was observed upon papain injection as well as during infection of *T. muris*, and eggs of *S. mansoni* (Perrigoue et al., 2009) (Sokol et al., 2009). These observations represented the potential of murine basophils to establish cognate interaction with T cells to initiate Th2 cell responses *in vivo*.

Further, studies have also indicated that DCs are not sufficient to induce Th2 responses against helminths and proteases due to lack of IL-4 secretion and incapability to phagocytize multicellular pathogens such as helminths. However, basophils were shown to capture and internalize soluble antigen as well as antigen-IgE immune complexes and to induce Th2 differentiation *in vitro*. Depletion of basophils in mice using a monoclonal antibody against FcεRI, resulted in a decline in both *Il4* mRNA and consequent impairment of protective immunity to *T. muris* (Perrigoue et al. 2009). Similarly, adoptive transfer of antigen-loaded MHC class II-sufficient basophils into MHC class II-

deficient mice demonstrated that basophils alone were sufficient for papain-induced Th2 responses *in vivo* (Sokol et al., 2009). Finally, adoptive transfer experiments by Nakanishi and colleagues showed that basophils pulsed with DNP-OVA and IgE-anti-DNP induce strong Th2 responses and produce OVA-specific IgE and IgG1 (Yoshimoto et al., 2009). Therefore, altogether these studies validated the role of murine basophils as APCs in mounting Th2 responses *in vitro* and *in vivo*.

On the contrary to these results, in a murine model of house dust mite-mediated allergy, Hammad et al., showed that inflammatory DCs but not basophils are necessary and sufficient for induction of Th2 immunity. To investigate role of basophils in allergen-induced Th2 responses, the authors used two different strategies for *in vivo* depletion of basophils by using antibodies to FcεRI (MAR-1) or to CD200R3 (Ba103). Upon allergen challenge of the mice, reduction in the naive CD4<sup>+</sup> T cell polarization, abolition of allergic airway inflammation and reduction in Th2 cytokines was observed, particularly when MAR-1 was used for basophil depletion but not upon depletion by Ba103 (Hammad et al., 20010). The authors discovered that this discrepancy occurred due to off-target depletion of a subset of DC expressing FcεRI that is majorly accounted for mediating T cell responses. This subset of DC could be non-specifically depleted by treatment with the MAR-1 antibody leading to discrepancy in observation. Further, the authors observed that this cell population positive for FcεRI<sup>+</sup> was also recruited to lymph node during allergen-challenge and found to express high level of MHC-II then basophils. These FcεRI<sup>+</sup> DX5<sup>-</sup> cells were positive for DC-specific marker CD11c and induced strong proliferation of T cells and increased level of Th2 cytokines when co-

cultured with T cells *in vitro*. However, FcεRI<sup>+</sup>DX5<sup>+</sup> basophils failed to take up the antigen and also could not induce T cell response.

The authors further identified this CD11c<sup>+</sup>FcεRI<sup>+</sup>DX5<sup>-</sup> subset as inflammatory DCs and also indicated that this subset is distinct from steady state DCs. They found that FcεRI is only expressed on monocyte-derived, inflammatory-type DCs induced by GM-CSF or IL-3 and not by steady state conventional DCs (Hammad et al., 2010). These results indicated that inflammatory DCs but not basophils are necessary and sufficient for induction of Th2 responses against house dust mite allergen. In conclusion, it is apparent that *in vivo* depletion of basophils should be carefully conducted as off-target depletion of inflammatory DCs might lead to bias results. Further, previous studies where basophils were demonstrated as potent APCs in mounting Th2 responses against helminth parasites and protease allergen had utilized MAR-1 antibody to deplete basophils *in vivo* (Sokol et al., 2009; Perrigoue et al. 2009). Also, adoptive transfer experiments conducted in these reports were based on basophils purification of FcεRI<sup>+</sup> cells (Yoshimoto et al., 2009) or by using DX5 beads (Sokol et al., 2009), in both cases purification could still obtain contaminating FcεRI<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup>DCs in purified cells (Hammad et al., 2009). Thus in view of these reports, role of basophils as APCs is still elusive and it is not clear whether inflammatory DCs have any role in development of Th2 environment against helminth infection or protease allergens as shown for basophils. However, as an alternative hypothesis, another group proposed that a Th2 response to cysteine proteases requires the co-operation between DCs and basophils (Tang et al., 2010).

In contrast to murine reports, studies by us and other groups (Sharma et al., 2013) (Eckl-

Dorna et al., 2012) {Kitzmuller, 2012 #156} {Dijkstra, 2012 #60} have strongly suggested that human basophils are incapable to act as professional APCs. I have demonstrated that human basophils lack the features of professional APCs and therefore alone can not induce T cell polarization (Sharma et al., 2013). I found that circulating basophils do not express antigen presenting molecules HLA-DR as well as co-stimulatory markers CD80 and CD86 required for efficient APC-mediated T cell activation and differentiation. Basophils remained negative for these markers at steady state condition as well as upon providing stimulation by an array of antigens including Asp f 1, allergen from *Aspergillus fumigatus*; Bet v 1, major birch allergen and Phl p 5, a major timothy grass pollen allergen (Sharma et al., 2013). Further, stimulation via TLR2 ligand and even anti-IgE-mediated activation could not induce HLA-DR and CD80, CD86 on human basophils. Finally, I reported that antigen-pulsed DCs and not basophils could induce IL-4 production from CD4<sup>+</sup> T cells in *in vitro* co-culture conditions.

APC-mediated CD4<sup>+</sup> T cell polarization implicates four different signals: signal zero at the interface of APCs and the antigen, signal 1 at the interface of antigen-loaded HLA-DR on APCs with T cell receptor-CD3 complexes on T cells, signal 2 that implicates the interaction of co-stimulatory molecules CD80/CD86 on APCs with CD28 of T cells and signal 3 in the form of CD4<sup>+</sup> T cell-polarizing cytokines secreted by APCs (Aimanianda et al., 2009). Although, human basophils secrete high amounts of Th2-polarizing cytokine IL-4, my results demonstrate that unlike other professional APCs, circulating human basophils lack signal 1 and signal 2 that are implicated in T cell polarization.

Additionally, my results were substantiated by studies conducted in birch pollen-allergic

patients (Kitzmüller et al. 2011; Eckl-Dorna et al., 2012; Poulson et al., 2012) which revealed that basophils from allergic patients are not capable to induce allergen-specific T cell responses upon secondary exposure of birch pollen allergen, Bet v 1. However, basophil depleted fraction of APCs that majorly contains CD11c<sup>+</sup> cells enhanced T cell proliferation in an *in vitro* co-culture system (Eckl-Dorna et al., 2012). Further, by using fluorescently-labeled Bet v 1, Kitzmüller et al. assessed whether basophils from birch pollen-allergic patients could bind and internalize the antigen. They found that basophils very efficiently bound Bet v 1 through IgE/FcεRI complexes on their surface. However, basophils did not internalize allergen and marginally expressed the proteins cathepsin S and invariant chain that are crucial for antigen presentation. The authors could not observe expression of antigen presenting HLA-DP, HLA-DQ and Co-stimulatory molecules CD80/CD86 and CD40 on either purified basophils or IL-3 plus IFN-γ treated basophils. However, marginal increase in HLA-DR was observed upon cytokine treatment. Bet v 1-pulsed basophils failed to act as APCs in co-culture experiments with Bet v 1-specific T-cell clones and could not induce T cell proliferation. These results further confirmed the inability of basophils to internalize, process and present antigen in context to MHC-II to induce T cell responses in healthy as well as in IgE-mediated allergy in humans.

Apart from allergic conditions, Charles et al., demonstrated that human basophils could express MHC-II molecules and therefore are capable to act as APCs in Th2-associated autoimmune condition such as SLE (Charles et al., 2010). However, Dijkstra et al. contradicted this observation, by showing that surface markers that are frequently used to

analyze basophils such as FcεRIα and CD123 are shared by plasmacytoid DC (pDC) and basophils. Therefore, based on phenotype CD123<sup>+</sup>CD304<sup>-</sup> as basophils and CD123<sup>+</sup>CD304<sup>+</sup> as pDC, the authors demonstrated in eight SLE patients that these are not basophils, but pDCs that identified MHC-II-expressing cells (Dijkstra et al., 2012).

Further, a recent finding showed that up to ~17% of human basophils could express MHC-II molecule and low level of co-stimulatory molecules CD80 and CD86, upon cytokine treatment with IL-3, IFN-γ and GM-CSF (Voskamp et al. 2013). Despite the confirmation of up-regulation of HLA-DR, HLA-DM, CD74 and Cathepsin S in MHC Class II-positive basophils, these basophils failed to induce antigen-specific T cell activation or proliferation (Voskamp et al. 2013). Thus, further confirmed the incompetence of human basophils to act as professional APCs.

Considering the reports from murine and human basophils, it is apparent that human and mice basophils could be different in various aspects. Therefore, further exploration is essential to establish the mechanism of Th2 responses in various immune micro-environment and the precise role of basophils and other APCs in this context.

## **2. Basophils in mediating Th17-associated responses**

In view of the evidences demonstrating basophils as a crucial player in several inflammatory, autoimmune and parasitic infection-associated conditions, I investigated whether human basophils are also associated with other T cells responses. In this context, a newly identified subset of T cells namely Th17, found to be associated with several autoimmune and inflammatory diseases, such as rheumatoid arthritis, SLE, multiple

sclerosis, psoriasis, inflammatory bowel disease (IBD), and allergy and asthma (Wilke et al., 2011). These cells express lineage specific transcription factor RORC and known to secrete other inflammatory mediators such as IL-17A, IL-17F, IL-21, IL-22, GM-CSF and CCL20 (Maddur et al., 2012). Th17 cells are associated in several inflammatory and autoimmune conditions where basophils are also reported as one the player in the development of pathologic conditions such as SLE, IBD, allergy and asthma. Thus, suggested the possibility for implication of basophil in Th17 responses. Recently, Mbow et al., reported that Th17 cells are associated with pathology in human schistosomiasis. They found elevated Th17 cells in peripheral blood of patients with schistosomiasis as well as in the liver and spleen of *Schistosoma mansoni*-infected mice (Mbow et al., 2013). However, the role of innate cells in initiation of these responses was not investigated. Several lines of evidences suggest implication of basophils in immune responses against helminth parasites including Schistosomes (Perrigoue et al., 2009). Therefore, I explored if human basophils or DCs, the known professional APC, are implicated in mediating Th17 responses against Schistosomes. I found that *S. mansoni* egg-treated DCs and not basophils significantly induced the expansion of IL-17<sup>+</sup> T cells in mixed lymphocyte reactions. Further, this induction was found to be associated with enhanced secretion of Th17-associated cytokines IL-17A, IL-17F and IL-22 from CD4<sup>+</sup> T cells that were cultured with antigen-pulsed DCs. However, *S. mansoni* egg-treated basophils did not alter the production of these cytokines (Sharma et al., 2014). Therefore, these results have clearly showed that despite known association of basophil responses in Schistosome infections, they are not implicated in mounting pathogenic Th17 responses.

However, earlier reports demonstrated the role of basophils in establishing protective immunity against schistosome infections, by promoting Th2 environment (Falcone et al., 1996) (Perrigoue et al., 2009). Therefore, altogether with these reports my results indicate that basophils could have role in promoting protective immunity against schistosome infections but their responses are not associated with Th17-implicated pathogenesis of disease (Mbow et al., 2013) (Sharma et al., 2014).

Another study by Wakahara et al., showed direct association of human basophils in enhancing Th17 responses upon interaction with CD4<sup>+</sup> memory T cells {Wakahara, 2012 #65} . In contrast, I could not detect any induction of Th17 responses when memory (CD4<sup>+</sup>CD45RO<sup>+</sup>) T cells were cultured with either resting basophils as well as IL-3 and FcεRI-stimulated basophils. The reasons for the discrepancies in the results are not clear. Differences in the serum source and stimulatory conditions could be the possible reasons. Considering my previous observation that indicate inability of human basophils to act as APCs, I explored role of basophils in a different experimental setup where along with basophils, CD4<sup>+</sup> CD45RO<sup>+</sup> memory T cells were cultured in the presence monocytes. I observed that TLR-agonist (peptidoglycan)-pulsed monocytes alone could significantly augment Th17 responses from memory T cells. However, presence of basophils did have any additive effect neither on expansion of IL-17<sup>+</sup> T cells nor on release of Th17-associated cytokines.

Based on their results and presence of basophils in the inflamed mucosal tissues, Wakahara et al., also suggested a role for basophils in the pathogenesis of inflammatory bowel disease (Wakahara et al., 2012). However, on the contrary, a recent report using

the colitis model demonstrates that basophils limit the disease activity in experimental murine colitis (Gomez et al., 2014). Also, due to failure of anti-IL-17A monoclonal antibody, the pathogenic role of Th17 cells in inflammatory bowel disease remains controversial (Hueber, et al., 2012). Together, my results indicate that basophils lack the ability to augment Th17 cell responses either directly or via APC. Therefore, increased activation and accumulation of Th17 cells in various inflammatory diseases implicate the repercussion of stimulation of these cells by innate cells other than basophils such as either monocytes or DCs. Altogether, our observations also signified the notion that basophil and Th17 cell responses in inflammatory diseases could be two independent but parallel events.

Recently, Gaudenzio et al, reported that human mast cells drive memory CD4<sup>+</sup> T cells toward IL-22 producers. It is well identified that mast cells share common features with basophils and often regarded as tissue resident counterpart of basophils. Both types of cells release various cytokines upon activation, including IL-4, IL-6, and IL-13; express common receptors, such as FcεRI, CD200R3, C3aR, C5aR, IL-3 receptor, IL-18 receptor, and IL-33 receptor; and release several inflammatory mediators, such as histamine and leukotrienes (Voehringer, 2013). IL-22 is associated with many chronic inflammatory conditions, including psoriasis and rheumatoid arthritis and enhanced production of IL-22 often being correlated with disease activity. Interestingly, IL-22 is known to be protective in other conditions such as inflammatory bowel disease, respiratory viral infection and in airway inflammatory diseases (Guo and Topham, 2010) (Taube et al., 2011). Considering, basophils as circulating counterpart of mast cells, I explored if like mast

cells, basophil could play a role in enhancing the production of IL-22 from CD4<sup>+</sup> T cells. My results elucidated that although basophils share several common properties with mast cells, unlike these cells, basophils do not possess the capacity to drive memory CD4<sup>+</sup> T cells toward IL-22 producers, either directly or through APCs.

Collectively, my observations indicated that basophils are not associated with the development of Th17-associated responses. In fact, basophils were failed to provide any additive impact on APC-mediated Th17 responses, which further corroborated my observation. However, in the context of IL-22 production, though my results indicated that basophils could not induce IL-22 production from CD4<sup>+</sup> T cells, the impact of basophils on other IL-22 producing cells such as NK cells remained to be explored.

### **3. Regulation of basophil function by CD4<sup>+</sup>CD25<sup>+</sup> Tregs**

Basophils are implicated in the pathogenesis of several Th2 –associated and B cell-mediated autoimmune and inflammatory conditions such as in lupus nephritis, chronic urticaria and eosinophilic esophagitis (Charles et al., 2010) (Kikuchi and Kaplan, 2001) (Noti et al., 2013). Altogether these reports highlighted the impact of dysregulated basophil functions on the outcome T and B cells immune responses leading to pathological conditions. Therefore, I sought to investigate whether CD4<sup>+</sup>CD25<sup>+</sup> Tregs that are well established for their immune-modulatory properties, could regulate basophils functions.

Tregs are known to be actively engaged in the maintenance of self-tolerance and immune homeostasis(Sakaguchi, 2004). *In vivo* studies demonstrated that depletion of Tregs could

lead to development of autoimmune and inflammatory conditions(Sakaguchi, 2004))(Sakaguchi et al., 2006) (Singh et al., 2001). Tregs have been reported to mediate suppressor functions on wide range of cellular targets that include CD4<sup>+</sup>, CD8<sup>+</sup> T cells, DCs, B cells, monocytes, macrophages, mast cells, NK and NKT cells (Bayry et al., 2007) (Azuma et al., 2003) (Miyara and Sakaguchi, 2007) (McNally et al., 2011).

However, my results elucidated that unlike above immune cells, human basophils are refractory for Treg-mediated suppression. I could not observe any inhibition in activation and histamine release upon stimulating Treg-educated-basophils via IL-3 and FcεRI-crosslinking. Expression of activation markers and level of histamine secretion was similar in basophils that were either cultured with Tregs or Tconv. These data thus indicated unresponsiveness of human basophils toward Treg-mediated suppressive mechanisms. Therefore, I then explored why the suppressive mechanisms of Tregs were not functional on basophils. Several mutually non-exclusive mechanisms of Tregs have been identified, some of which are contact-dependent and others are contact-independent and are mediated through cytokines(Sakaguchi et al., 2009). The contact-dependent functions of Tregs are mediated mainly via CTLA-4 (cytotoxic T lymphocyte antigen 4) and LAG3 (lymphocyte activation gene 3) which interact with CD80/86 and HLA-DR respectively (Huang et al., 2004) (Liang et al., 2008) (Wing et al., 2008) (Onishi et al., 2008). In addition, granzyme A and Fas-FasL interactions were also implicated in exerting Treg-mediated cytotoxic effects on target cells (Sakaguchi et al., 2010) (Grossman et al., 2004) (Cao et al., 2007)

However, consistent with the earlier observations, I found absence of antigen presenting molecule HLA-DR and co-stimulatory CD80 and 86 on resting as well as on stimulated basophils. This observation itself ruled out possibility of contact-dependent suppression via LAG3 and CTLA-4. I have also measured the viability of basophils cultured with either Tregs or Tconv, by annexin V and propidium iodide staining. However, no significant differences were observed in the level of viability of control basophils and T cell-co-cultured basophils. These results clarified that Tregs could not induce contact-dependent suppression of basophils as well as cytotoxic effects on these cells.

Previous studies have demonstrated that Tregs-mediated suppressive functions are also mediated by cytokines and in particular TGF- $\beta$  and IL-10 (Andersson et al., 2008; Sakaguchi et al., 2010). IL-10 is reported to inhibit: T cell proliferation and cytokine release (Del Prete et al., 1993), DC maturation and function (De Smedt et al., 1997), and pro-inflammatory cytokine release from monocytes (de Waal Malefyt et al., 1991). Whereas, TGF-  $\beta$  was shown to suppress IL-8 release and migration of neutrophils (Smith et al., 1996), inhibition of DCs and inhibition of B cell antibody production and membrane immunoglobulin expression (Kehrl et al., 1999). Thus, the suppressive effects mediated by these two cytokines are well established on various immune cells.

Therefore, I first assessed if basophils express receptors for these two cytokines and found that basophils lack receptor for TGF- $\beta$  but substantial proportion ( $36 \pm 5\%$ ) of basophils were found to express IL-10 receptor (IL-10R). Surprisingly, I could not observe inhibitory effect of IL-10 on basophils when stimulated with anti-IgE. Also, expression of activation markers was found similar on IL-10R<sup>+</sup> and IL-10R<sup>-</sup> subsets of

basophils. Altogether, these observations comprehensively elucidated the resistance of human basophils to Treg-mediated suppression.

Further, Tregs were demonstrated to inhibit FcεRI-induced degranulation of murine mast cells, the tissue-resident counterpart of basophils, in an OX40-OX40L-dependent manner (Gri et al., 2008) (Ganeshan and Bryce, 2012) whereas TGF-β/TGF-βR interaction was found to be necessary for enhancing IL-6 production from mast cells (Ganeshan and Bryce, 2012). Tregs also inhibited FcεRI expression and its signaling in murine mast cells (Kashyap et al., 2008). Although these results are not yet confirmed in humans, our results showed that Tregs did not affect degranulation process of human basophils as measured by histamine release and also did not inhibit FcεRI expression. Despite human basophils share several common features with mast cells, they do display distinct features. Unlike basophils, human mast cells express co-stimulatory and antigen-presenting molecules and induce activation and proliferation of CD4<sup>+</sup> T cells (Gaudenzio et al., 2013) (Sharma et al., 2013) (Kitzmuller et al., 2012). Based on present data, we suggest that as compared to mast cells, human basophils also display discrete features of regulation.

A recent report showed that chronic helminth infection in mice reduced basophil responsiveness to IgE cross-linking in an IL-10-dependent manner (Larson et al., 2012). However, as I and other researchers demonstrated, mice and human basophils display distinct differences in their surface phenotype and functions. In addition, human basophils were also unresponsive to IL-10 and did not inhibit activation of basophils upon IgE-cross-linking. This altogether validates the functional distinction between

human and mice basophils. Alternatively, as IL-10 is known to have suppressive effects on other immune cells and the fact that basophils receive activation-associated signals both from innate and adaptive immune cells, the observed IL-10-dependent suppression of murine basophil response could be indirect rather than its direct effect on basophils

## **Perspectives**

I demonstrated that circulating human basophils lack the ability to act as APCs, which is in contrast to observations made in murine models. It is important to mention that studies on antigen-presenting and Th2 polarizing capabilities of murine basophils were performed on the cells that were recruited to secondary lymphoid organs such as lymph nodes and spleen. However, my findings on human basophils are mainly from circulating basophils. Therefore it is not known whether microenvironment of secondary lymphoid organs influence the phenotype and functions of basophils. Owing to the fact that immune-microenvironment has great impact on phenotypic characteristics as well as on functional behavior of the cells, it is critical to examine the antigen-presenting capabilities of human basophils derived from lymphoid organs such as spleen, lymph nodes and tonsils.

In the context of regulation of basophil function, I could not observe Treg-mediated functional suppression of human basophils. I have also identified the reasons for unresponsiveness of basophils toward suppressive mechanism of Tregs. However, regulation of basophils by other cellular components of immune system has not been explored much. In this regard, CD4<sup>+</sup> helper T cells are known for their critical role in the education of various immune cells. Therefore, it would be interesting to explore how different CD4<sup>+</sup> T cell subsets viz. Th1, Th2 and Th17 could impact and regulate the basophil function.

Further, as basophils are known to have an impact on T and B cell responses, exploration for their role in the pathogenesis of T cells or B cells-associated autoimmune conditions and the effect of therapeutic interventions on basophil function, would be of great importance. In this regard, Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal pooled immunoglobulin G (IgG) obtained from the plasma of several thousand healthy donors and is widely used in the treatment of various autoimmune and inflammatory diseases including Kawasaki disease, inflammatory myopathies, Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy and myasthenia gravis. The beneficial effects of IVIg in diseases are attributed due to several mutually non-exclusive mechanisms that include inhibition of activation and functions of innate immune cells such as dendritic cells (DCs), monocytes, macrophages and neutrophils; inhibition of pathogenic effector T cells such as Th1 and Th17 cells; expansion of regulatory T cells (Tregs) and modulation of B cell responses. However, the effect of immune-modulatory properties of IVIg on basophils in the autoimmune conditions is not much explored. Recently, in murine model, it was demonstrated that anti-inflammatory effect of IVIg is mainly mediated by a small fraction of antibodies that contain terminal  $\alpha$ 2,6-sialylated glycans at Asn297 in Fc (Fragment crystallizable) portion of antibodies. These  $\alpha$ 2, 6-sialylated Fc fragments interact with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin-positive (DC-SIGN<sup>+</sup>) innate cells to release IL-33, leading to expansion of IL-4-

producing basophils. However, translational significance of these observations in human autoimmune patients is lacking. It would be of great importance to investigate whether high-dose IVIg therapy induces IL-33 production in autoimmune patients and if this in turn could mediate basophil expansion and IL-4 responses.

Further, considering the implication of basophils in the pathogenesis of other autoimmune diseases such as lupus nephritis, it is not known how basophils enter into inflammatory cascade. In this regard, it is essential to understand the factors responsible for the initial activation of basophils in early disease flare. Therefore, considering essential role of CD4<sup>+</sup> T cells in providing help to other immune cells during immune responses, investigation for the role of CD4<sup>+</sup> T cells on basophil activation and function could be of interest. Since, CD4<sup>+</sup> T cells are the source of IL-3, the cytokine majorly responsible for basophil hematopoiesis, survival and priming; it is likely that activated CD4<sup>+</sup> T cells in autoimmune conditions might act as source of initial stimuli to basophils. The investigations focused on comparison of basophil activation mediated by healthy donor CD4<sup>+</sup> T cells vs. CD4<sup>+</sup> T cells from lupus patients could provide more insight on basophils and CD4<sup>+</sup> T cell cross talk in autoimmune conditions.

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# Annexes

## **Publications:**

- Article 1:** **Sharma M**, Schoindre Y, Hegde P, Saha C, Maddur MS, Stephen-Victor E, Gilardin L, Lecerf M, Bruneval P, Mouthon L, Benveniste O, Kaveri SV, Bayry J. Intravenous IL-33 is insufficient to mediate basophil expansion in autoimmune patients. *Scientific Reports*. 2014,11; 4:5672.
- Article 2:** Maddur MS, **Sharma M**, Hegde P, Stephan EV, Pulendran B, Kaveri SV and Bayry J. Human B cells induce dendritic cell maturation in a contact-dependent manner and favor Th2 polarization by inducing OX-40 ligand. *Nature Communications*. 2014, 9(5): 4092.
- Article 3:** **Sharma M**, Schoindre Y, Gilardin L, Saha C, Benveniste O, Kaveri SV, Bayry J. Interferon  $\alpha$  inhibition by intravenous immunoglobulin is independent of modulation of plasmacytoid dendritic cell population in the circulation. *Arthritis Rheumatology* 2014 66(8): 2308-9.
- Article 4:** **Holla S, Sharma M\***, Vani J, Kaveri SV, Balaji KN, Bayry J. GM-CSF along with IL-4 but not alone is indispensable for the differentiation of human dendritic cells from monocytes. 2014. *Journal of Allergy and Clinical Immunology*. 133 (5): 1500-02. (\* Shared first authorship)
- Article 5:** **Trinath J, Hegde P, Sharma M\***, Maddur MS, Rabin M, Vallet JM, Magy L, Balaji KN, Kaveri SV, Bayry J. Intravenous immunoglobulin expands regulatory T cells via induction of cyclooxygenase-2-dependent prostaglandin E2 in human dendritic cells. 2013. *Blood*. 122(8): 1419-27 (\*Shared first authorship)
- Article 6:** Othy S, Hegde P, Topcu S, **Sharma M**, Maddur MS, Lacroix-Desmazes S, Bayry J, Kaveri SV. Intravenous gammaglobulin inhibits encephalitogenic potential of pathogenic T cells and interferes with their trafficking to the central nervous system, implicating sphingosine-1 phosphate receptor 1–mammalian target of rapamycin axis. 2013. *Journal of Immunology*. 190(9):4535-41
- Article 7:** Maddur MS, **Sharma M**, Hégde P, Lacroix-Desmasez S, Kaveri SV, Bayry J. Inhibitory effect of IVIG on IL-17 production by Th17 cells is independent of anti-IL-17 antibodies in the immunoglobulin preparations. 2013. *Journal of Clinical Immunology*. 33(1):62-66.
- Article 8:** Vani J, **Sharma M**, Shaila MS, Kaveri SV, Bayry J. Impact of gp120 on dendritic cell-derived chemokines: relevance for the efficacy of gp120-based vaccines for HIV-1. 2012. *Clinical and Vaccine Immunology*. 19(8):1335-6.

**Article 9:** Maddur MS, Hegde P, Sharma M, Kaveri SV, Bayry J. B cells are resistant to immunomodulation by 'IVIg-educated' dendritic cells.2011. Autoimmunity Reviews. 11(2):154-6.

**Editorial article :** Sharma M, Kaveri SV, Bayry J Th17 cells, pathogenic or not? TGF-b3 imposes the embargo. 2013. Cellular and Molecular Immunology. 10(2):101-102.



OPEN

SUBJECT AREAS:

AUTOIMMUNITY

DENDRITIC CELLS

IMMUNOTHERAPY

Received  
25 April 2014

Accepted  
26 June 2014

Published  
11 July 2014

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# Intravenous immunoglobulin-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients

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**Intravenous immunoglobulin (IVIg) is used in the therapy of various autoimmune and inflammatory diseases. Recent studies in experimental models propose that anti-inflammatory effects of IVIg are mainly mediated by  $\alpha 2,6$ -sialylated Fc fragments. These reports further suggest that  $\alpha 2,6$ -sialylated Fc fragments interact with DC-SIGN<sup>+</sup> cells to release IL-33 that subsequently expands IL-4-producing basophils. However, translational insights on these observations are lacking. Here we show that IVIg therapy in rheumatic patients leads to significant raise in plasma IL-33. However, IL-33 was not contributed by human DC-SIGN<sup>+</sup> dendritic cells and splenocytes. As IL-33 has been shown to expand basophils, we analyzed the proportion of circulating basophils in these patients following IVIg therapy. In contrast to mice data, IVIg therapy led to basophil expansion only in two patients who also showed increased plasma levels of IL-33. Importantly, the fold-changes in IL-33 and basophils were not correlated and we could hardly detect IL-4 in the plasma following IVIg therapy. Thus, our results indicate that IVIg-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients. Hence, IL-33 and basophil-mediated anti-inflammatory mechanism proposed for IVIg might not be pertinent in humans.**

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal pooled immunoglobulin G (IgG) obtained from the plasma of several thousand healthy donors. High-dose IVIg (1–2 g/kg) is widely used in the treatment of various autoimmune and inflammatory diseases including Kawasaki disease, idiopathic thrombocytopenic purpura, Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, autoimmune blistering diseases, inflammatory myopathies, graft versus host disease and others<sup>1–4</sup>. The cellular and molecular mechanisms of action of IVIg in these diverse diseases remain incompletely understood. However, available evidence both from experimental and clinical studies provide an indicator that IVIg could benefit these diverse diseases via several mutually non-exclusive mechanisms<sup>2,5–10</sup>. These mechanisms include inhibition of activation and functions of innate immune cells such as dendritic cells (DCs), monocytes, macrophages and neutrophils; inhibition of pathogenic effector T cells such as Th1 and Th17 cells; expansion of regulatory T cells (Tregs); modulation of B cell responses; and inhibition of complement pathways. In addition, IVIg has been shown to inhibit inflammatory cytokines and to augment anti-inflammatory molecules such as IL-10 and IL-1 receptor antagonist<sup>11–21</sup>.

IgGs are glycoproteins and contain fragment antigen-binding (Fab) regions that recognize antigens, and fragment crystallizable (Fc) regions that exert effector functions upon binding to Fc $\gamma$  receptors. The Fc fragments are glycosylated at Asn297 and recent studies in animal models advocate that anti-inflammatory effects of IVIg



are mediated by a small fraction of antibodies that contain terminal  $\alpha$ 2,6-sialylated glycans at Asn297. It was proposed that  $\alpha$ 2,6-sialylated Fc fragments interact with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin-positive (DC-SIGN<sup>+</sup>) innate cells to release IL-33, which subsequently expands IL-4-producing basophils<sup>22</sup>. However, translational insights on these observations are lacking. Therefore, we investigated whether high-dose IVIg therapy induces IL-33 production in autoimmune patients, which in turn would mediate basophil expansion and IL-4 responses.

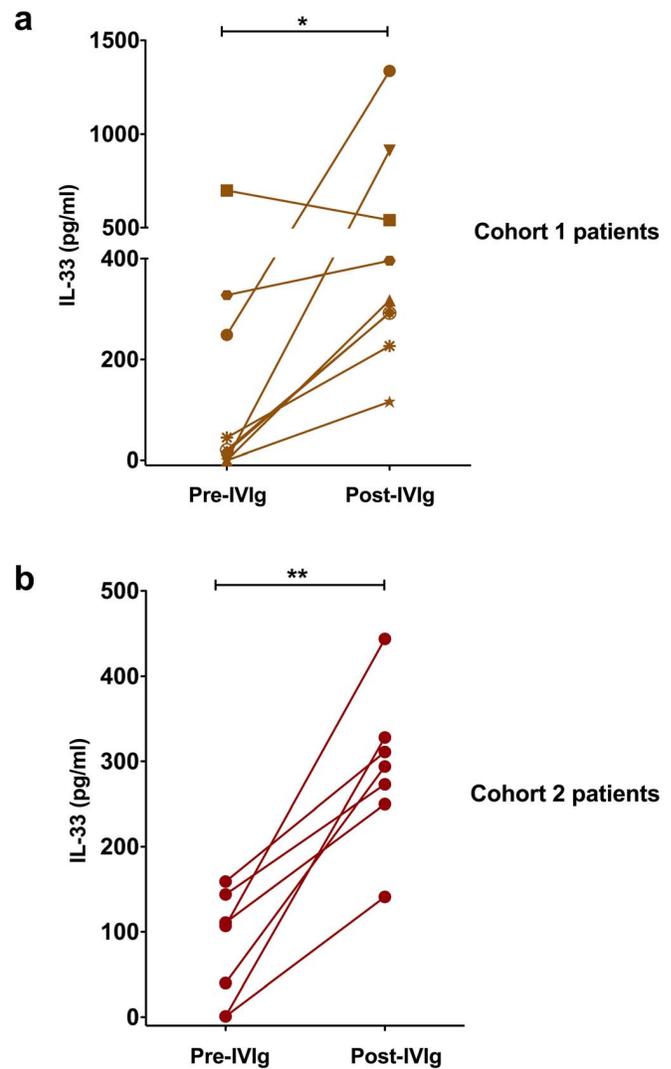
## Results

**IVIg therapy induces IL-33 in autoimmune patients.** Previous work on the role of IL-33 in IVIg-mediated anti-inflammatory effects was performed in K/BxN serum-induced murine arthritis model. It should be noted that IVIg is not recommended for rheumatoid arthritis due to its inefficacy to relieve inflammation<sup>4</sup>. Therefore, K/BxN serum-induced murine arthritis model might not provide factual image of the mechanisms of IVIg in autoimmune patients. Earlier studies have indicated that IVIg therapy benefits patients with inflammatory myopathies<sup>1,4</sup>. Therefore, by using heparinized blood samples of these patients (cohort 1 patients), we first investigated the repercussion of IVIg therapy on the induction of IL-33. We found that, out of nine patients, six had minimal level of plasma IL-33 prior to IVIg therapy. The pre-IVIg plasma level of IL-33 was in the range of  $150.75 \pm 79.52$  pg/ml ( $n = 9$ ) (Fig. 1a). Following IVIg therapy, with an exception of one patient, all remaining patients had significant raise in plasma IL-33 and was in the range of  $492.23 \pm 130.30$  pg/ml ( $n = 9$ ) (Fig. 1a). However, the increase in IL-33 following IVIg therapy was heterogeneous and was varying from 1.2 to 911-fold.

To confirm these results, we analyzed the plasma samples from another cohort of patients with inflammatory myopathies ( $n = 4$ ) or anti-neutrophil cytoplasmic antibody-associated vasculitis ( $n = 3$ ) (cohort 2 patients). Importantly, these patients also showed significant increase in plasma IL-33 following IVIg therapy (Fig. 1b) thus confirming the results obtained with cohort 1 patients. The pre-IVIg plasma level of IL-33 was  $80.43 \pm 24.93$  pg/ml ( $n = 7$ ) that increased to  $291.58 \pm 34.40$  pg/ml following IVIg therapy. Together, these results indicate that irrespective of pathologies, IVIg therapy in patients leads to increased plasma level of IL-33.

**IVIg-induced IL-33 is not associated with an expansion of basophils.** Basophils play a crucial role in the induction of Th2 responses<sup>23,24</sup>. Recent data from K/BxN serum-induced murine arthritis model suggest that IVIg-induced IL-33 promotes basophil expansion<sup>22</sup>. Therefore, we investigated changes in the circulating basophils following IVIg therapy in cohort 1 patients. Basophils were identified based on the expression of Fc $\epsilon$ RI and CD203c (Fig. 2a)<sup>25</sup>. In contrast to the results from murine model, we found that IVIg therapy leads to basophil expansion only in two patients who also showed increased plasma level of IL-33 (Fig. 2b). In other patients, basophils were either declined or unaltered. The changes in the proportion of basophils in the circulation following IVIg therapy were not statistically significant. Importantly, the fold-changes in IL-33 and basophils were not correlated (Fig. 2c). Also contrary to previous report<sup>22</sup>, we could hardly detect IL-4 in the plasma of patients following IVIg therapy. Thus, these results demonstrate that IVIg therapy in patients does not lead to an expansion of basophils. Of note, a recent data from murine models of collagen antibody-induced arthritis and K/BxN serum transfer arthritis also reveal that therapeutic effect of IVIg is independent of sialylation and basophils<sup>26</sup>.

**DC-SIGN-positive human innate cells do not produce IL-33 upon IVIg exposure.** DC-SIGN<sup>+</sup> innate cells (or SIGN-R1<sup>+</sup> cells in the murine spleen) were proposed to produce IL-33 upon interaction

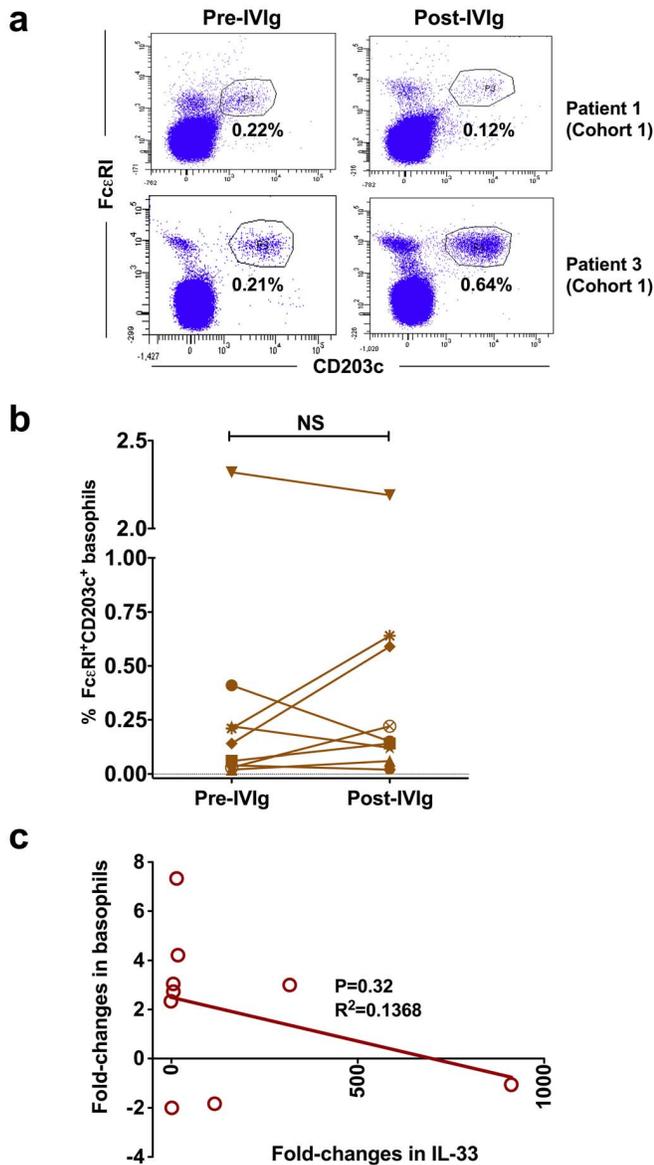


**Figure 1 | Consequence of IVIg therapy in autoimmune patients on the plasma level of IL-33.** (a) Heparinized blood samples were obtained from nine patients with inflammatory myopathies (Cohort 1 patients) before (Pre-IVIg) and 2-3 days after initiation of IVIg therapy (Post-IVIg). IL-33 (pg/ml) in the plasma was measured by ELISA. Each symbol in the graph represents individual patient. (b) IL-33 in the plasma of four inflammatory myopathies and three anti-neutrophil cytoplasmic antibody-associated vasculitis patients (Cohort 2 patients) before and post-IVIg therapy. The statistical significance as determined by two-tailed Student-t-test is indicated, where \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

with  $\alpha$ 2,6-sialylated Fc fragments of IVIg<sup>22</sup>. By generating humanized DC-SIGN-transgenic mice, the authors found that these transgenic mice express DC-SIGN on DCs, macrophages and monocytes in the blood, bone marrow and spleen. Importantly, higher percentage of monocytes in these transgenic mice expressed DC-SIGN<sup>22</sup>.

We analyzed the expression of DC-SIGN in human myeloid cells. Contrary to humanized DC-SIGN-transgenic mice, circulating human monocytes did not express DC-SIGN whereas its expression on macrophages was restricted to M2 type macrophages wherein up to 28% cells were positive for DC-SIGN. We could observe high expression of DC-SIGN ( $\approx 100\%$ ) only in monocyte-derived DCs (Mo-DCs) (Fig. 3a). In the human spleen, up to 24% splenocytes were positive for DC-SIGN (Fig. 3b).

Therefore, we explored if Mo-DCs secrete IL-33 upon IVIg treatment. In contrast to proposition by Ravetch and colleagues, we could

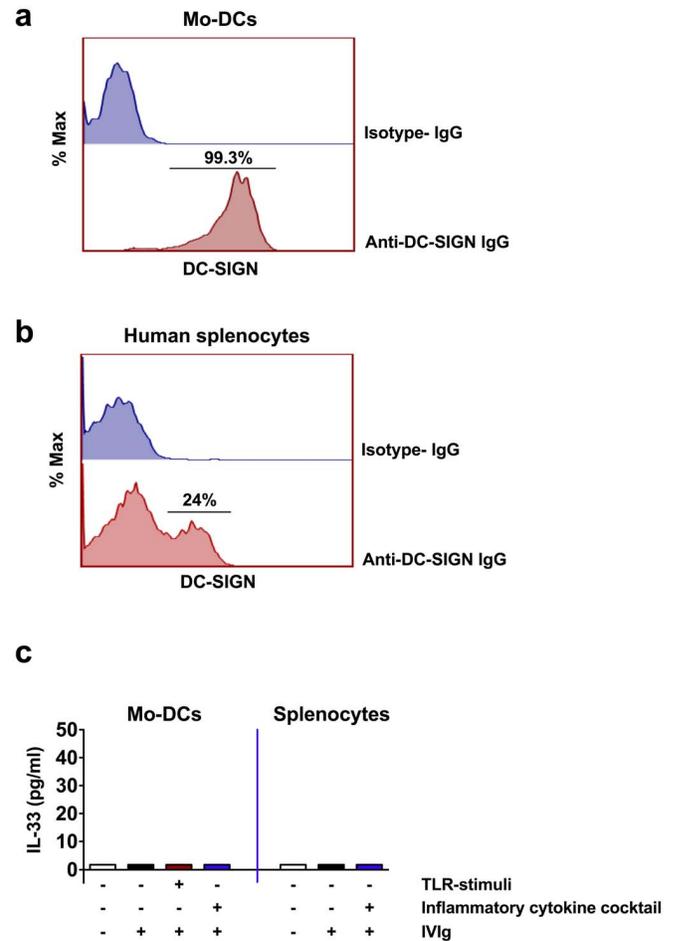


**Figure 2** | Changes in the proportion of circulating basophils of autoimmune patients following IVIg therapy. Heparinized blood samples were obtained from cohort 1 patients with inflammatory myopathies before (Pre-IVIg) and 2–3 days after initiation of IVIg therapy (Post-IVIg). (a) Representative dot-plots showing basophils from cohort 1 patients gated positive for FcεRI and CD203c (b) Modulation of circulating basophils following IVIg therapy (n = 9). Basophils were analyzed in the whole blood by flow cytometry. The statistical significance as determined by two-tailed Student-t-test is indicated, where NS, non-significant. (c) The correlation between fold-changes in IL-33 and basophils following IVIg therapy.

detect secreted IL-33 from IVIg-exposed DC-SIGN<sup>+</sup> Mo-DCs neither under non-inflammatory nor under inflammatory conditions (Fig. 3c). Similarly, despite the presence of DC-SIGN<sup>+</sup> cells in the spleen, human splenocytes did not produce detectable levels of IL-33 upon IVIg exposure both under inflammatory and non-inflammatory conditions (Fig. 3c).

## Discussion

Our results demonstrate that IVIg therapy induces IL-33 in autoimmune patients thus confirming the previous observation made in mice. However, IL-33 was not contributed either by splenic



**Figure 3** | Effect of IVIg on the IL-33 production from DC-SIGN<sup>+</sup> human innate cells. (a and b) Histograms showing the expression of DC-SIGN by healthy donor's monocyte-derived human dendritic cells (Mo-DCs) and splenocytes. (c) IVIg does not induce IL-33 from DC-SIGN<sup>+</sup> human innate cells. Mo-DCs or human splenocytes (n = 5 donors) were exposed to IVIg either under non-inflammatory conditions or under inflammatory conditions (TLR-stimuli or inflammatory cytokine cocktail) for 48 hours. IL-33 in the culture supernatants was analyzed by ELISA.

DC-SIGN<sup>+</sup> cells or myeloid DCs. Also, the amount of IL-33 induced in the patients was not sufficient to expand basophils. It should be noted that the quantity of IL-33 protein induced in the mice following IVIg treatment was not presented in the previous report. In addition, significant amount of data on IVIg was indirect rather than direct demonstration of IVIg-mediated regulation of cytokine network<sup>22</sup>. Authors showed that IVIg induces about 12-fold increase in IL-33 mRNA level. However, the contribution of this increased IL-33 mRNA towards IL-33 protein is not clear. Considering five liters as total blood volume in adults, our results show that IVIg induces  $\approx 2460 \pm 650$  ng of IL-33 (based on the data from cohort 1 patients). However, to demonstrate the role of IL-33 in IVIg-mediated anti-inflammatory effects, Anthony *et al.*, injected 400 ng of IL-33 for four consecutive days<sup>22</sup>. As mouse weighing 25 g would have  $\approx 1.5$  ml of blood, based on the IL-33 data from patients, we could infer that the amount of exogenous IL-33 injected into the mice represents at least 540-times excess of IL-33 that otherwise induced by IVIg. This might explain why IVIg failed to induce expansion of basophils in the patients. Although in our study, patients' sample size was small, we included diseases such as inflammatory myopathies and vasculitis that were shown to benefit from IVIg therapy. Further investigations in a larger number of patients should confirm these observations.



Table 1 | Summary of data for autoimmune rheumatic patients

Cohort 1 patients						
Number	Disease	Age (years)	Sex	IVIg	Additional treatments	
1	Polymyositis	59	F	CLAIRYG <sup>®</sup> 1 g/kg	Methylprednisolone	
2	Anti-SRP associated necrotizing myopathy	27	F	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
3	Anti-HMGCR associated necrotizing myopathy	62	F	CLAIRYG <sup>®</sup> 0.5 g/kg	Prednisone, Methotrexate	
4	Anti-HMGCR associated necrotizing myopathy	61	F	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
5	Dermatomyositis	52	F	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
6	Polymyositis associated with mixed connective tissue disease and Sjögren's syndrome	41	F	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
7	Anti-SRP associated necrotizing myopathy	40	M	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
8	Anti-Mi2 associated unclassified myositis	30	M	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
9	Polymyositis and probable associated Sjögren's syndrome	70	F	CLAIRYG <sup>®</sup> 1 g/kg	Prednisone, Methotrexate	
Cohort 2 patients						
Number	Disease	Age (years)	Sex	IVIg	Additional treatments	
1	Dermatomyositis	22	F	TEGELINE <sup>®</sup> 1g/kg	Prednisone, Mycophenolate mofetil	
2	Polymyositis	42	M	TEGELINE <sup>®</sup> 1g/kg	Prednisone, Methotrexate	
3	Dermatomyositis	35	M	TEGELINE <sup>®</sup> 1g/kg	Prednisone	
4	Polymyositis	46	F	TEGELINE <sup>®</sup> 1g/kg	Prednisone, ciclosporin	
5	Microscopic polyangiitis	61	F	TEGELINE <sup>®</sup> 1g/kg	Prednisone	
6	Wegener's granulomatosis	62	M	TEGELINE <sup>®</sup> 1g/kg	None	
7	Microscopic polyangiitis	61	M	TEGELINE <sup>®</sup> 1g/kg	Prednisone, Mycophenolate mofetil	

SRP, Signal Recognition Particle; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

The role of Fc-sialylation, DC-SIGN and Fc $\gamma$  receptor IIB (Fc $\gamma$ RIIB) in the anti-inflammatory effects of IVIg has been debated recently by several groups<sup>27</sup>. Mice and humans show wide variations in the expression pattern of Fc $\gamma$ Rs, and the phenotype and anatomical distribution of innate cells. Unlike mice, human innate cells express both activating Fc $\gamma$ RIIA and inhibitory Fc $\gamma$ RIIB. Therefore, the proposition that IVIg enhances Fc $\gamma$ RIIB on effector macrophages of mice without having corresponding data on Fc $\gamma$ RIIA might provide a biased picture on the mechanisms of IVIg. Gene array analysis could not confirm IVIg-mediated up-regulation of Fc $\gamma$ RIIB in the patients with Kawasaki disease<sup>28</sup>. In line with this report, another recent study failed to demonstrate enhanced expression of Fc $\gamma$ RIIB on monocytes following IVIg therapy in children with immune thrombocytopenia<sup>29</sup>. Also, Fc $\gamma$ R polymorphisms did not predict response to IVIg in myasthenia gravis<sup>30</sup>. Although DC-SIGN promoter -336 A/G (rs4804803) polymorphism was associated with susceptibility of Kawasaki disease, this variant was found to be not associated with the occurrence of IVIg resistance<sup>31</sup>. Of note, treatment response in Kawasaki disease is apparently associated with sialylation levels of endogenous IgG but not therapeutic IVIg<sup>32</sup>. All these data thus questions the relevance of DC-SIGN-Fc $\gamma$ RIIB pathway of anti-inflammatory mechanisms of IVIg in humans.

Several recent studies have challenged the concept of  $\alpha$ 2,6-sialylated Fc fragments-mediated anti-inflammatory mechanism of IVIg both in experimental models and in humans. IVIg could inhibit human Th17 cell differentiation and expansion independent of antigen presenting cells and hence independent of interaction of DC-SIGN and  $\alpha$ 2,6-sialylated Fc fragments<sup>13–15</sup>. Also, F(ab')<sub>2</sub> fragments of IVIg exerted similar effects thus pointing towards dispensability of  $\alpha$ 2,6-sialylated Fc fragments in mediating the suppression of Th17 cells. We have demonstrated that DC-SIGN and  $\alpha$ 2,6-sialylated Fc fragment interaction is dispensable for the anti-inflammatory activity of IVIg on human DCs<sup>33</sup>. F(ab')<sub>2</sub> fragments but not Fc fragments of IVIg were shown to mediate Treg expansion by inducing cyclooxygenase-2-mediated prostaglandin E2 secretion in human myeloid DCs and was dependent in part on DC-SIGN<sup>19</sup>. Similarly, sialylation-

enriched F(ab')<sub>2</sub> fragments could inhibit interferon- $\alpha$  production from toll-like receptor (TLR)7 and TLR9 stimulated human plasmacytoid DCs, although sialic acid itself was not required<sup>34</sup>.

In the previous reports, Ravetch and colleagues enriched sialic acid-containing IgG-Fc by using sialic acid-specific lectin *Sambucus nigra* agglutinin-based affinity fractionation<sup>22,35–37</sup>. However, by using same fractionation method, Guhr *et al.*, showed that IVIg fractions depleted for the sialylated antibody fraction exert benefits in a murine model of passive-immune thrombocytopenia similar to that of intact IVIg. However, sialic acid-enriched IVIg fraction failed to enhance platelets count in this model<sup>38</sup>. Similar sialic-acid independent anti-inflammatory mechanisms were also reported in murine herpes simplex virus encephalitis model<sup>39</sup>. Further, Käsermann and colleagues showed that lectin fractionation of IVIg results in increased sialylation of Fab fragments but not Fc fragments. By using human whole blood stimulation assay either with lipopolysaccharide or phytohaemagglutinin, they further showed that anti-inflammatory effects of IVIg is associated with F(ab')<sub>2</sub> fraction of IVIg<sup>40</sup>. In animal models of immune thrombocytopenia and multiple sclerosis, the beneficial effects of IVIg were independent of Fc or F(ab')<sub>2</sub> -sialylation and Fc $\gamma$ RIIB<sup>41–44</sup>. Based on these results, it was suggested that genetic background of the mice and dose of IVIg are the critical factors that determine the role of Fc $\gamma$ RIIB in IVIg-mediated beneficial effects. In line with these observations, two studies have failed to demonstrate the direct interaction between sialylated IgG Fc fragment and DC-SIGN<sup>45,46</sup>. These data thus point out that  $\alpha$ 2,6-sialylated Fc fragment-DC-SIGN-Fc $\gamma$ RIIB mechanism merely represents one of the several anti-inflammatory mechanisms of IVIg that were reported. Therefore, this anti-inflammatory pathway of IVIg might be operational in certain pathologies and experimental models and might not be considered as a universal mechanism.

It was proposed that in humanized DC-SIGN-transgenic mice, DC-SIGN<sup>+</sup> innate cells such as monocytes, macrophages and DCs produce IL-33 upon interaction with  $\alpha$ 2,6-sialylated Fc fragments of IVIg<sup>22</sup>. Recent reports show that IL-33 is an important player for the promotion of Th2 responses and activated DCs are one of the sources of this cytokine<sup>47,48</sup>. We found that unlike monocytes from huma-



nized DC-SIGN-transgenic mice that were highly positive for DC-SIGN, human monocytes hardly express DC-SIGN. Further, human Mo-DCs despite expressing DC-SIGN, failed to produce IL-33 when exposed to IVIg either under non-inflammatory or inflammatory conditions. In wild type mice, it was suggested that  $\alpha$ 2,6-sialylated Fc fragments bind to SIGN-R1 expressed on splenic marginal zone macrophages<sup>35</sup>. Marginal zone macrophages are absent in human spleen and data from humans show that spleen is dispensable for the anti-inflammatory effects of IVIg. In line with this concept, by using a passive model of induced immune thrombocytopenia, it was shown that IVIg is fully functional in splenectomized mice although this report supported the sialic acid and SIGN-R1-dependent mechanisms of IVIg<sup>49</sup>. We found that despite the presence of DC-SIGN<sup>+</sup> innate cells in the human spleen, IVIg could not induce IL-33 from the splenocytes. All these data indicate that spleen and DC-SIGN<sup>+</sup> cells are dispensable for IVIg-mediated IL-33 induction in humans. Thus, the source of IL-33 in humans following IVIg therapy remains elusive. As IVIg is known to cause apoptosis of cells, we suggest that secondary necrosis of late stage apoptotic cells could release IL-33<sup>50–52</sup>. This process might depend on the signals provided by anti-Fas IgG or anti-Siglec IgG in the IVIg preparations rather than the repercussion of interaction of  $\alpha$ 2,6-sialylated Fc fragments with DC-SIGN<sup>53,54</sup>. In addition, IL-33 is also constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*<sup>55</sup>.

## Methods

**Patients.** All experiments were performed in accordance with relevant guidelines and regulations. We obtained heparinized blood samples of nine patients (cohort 1 patients) with inflammatory myopathies (Table 1). Patients were aged  $49.1 \pm 15.2$  years and include two men. Blood samples were obtained before and 2–3 days following initiation of IVIg therapy (CLAIRYG<sup>®</sup>, Laboratoire Français du Fractionnement et des Biotechnologies, France). Informed consent was obtained from all the patients. The study was approved by CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris. In addition, we also analyzed plasma samples of seven rheumatic patients (cohort 2 patients) before and 2–3 days post-IVIg therapy (TEGELINE<sup>®</sup>, Laboratoire Français du Fractionnement et des Biotechnologies, France). The patients were aged  $47 \pm 5.8$  years (four men) and include inflammatory myopathies and anti-neutrophil cytoplasmic antibody-associated vasculitis (Table 1).

**Analysis of basophils.** Red blood cells (RBCs) from heparinized blood samples of cohort 1 patients were depleted by using HetaSepTM (Stemcell Technologies Sarl, France) and nucleated cell suspension was obtained. Basophils were analyzed in RBC-depleted cell suspension by flow cytometry (LSR II, BD Biosciences, France) using fluorochrome-conjugated monoclonal antibodies to Fc $\epsilon$ R1 (Miltenyi Biotec, France) and CD203c (eBioscience, France). Data were analyzed by FACSDiva™ software (BD Biosciences).

**Generation of monocyte-derived DCs.** Buffy coats from the healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang (EFS), Paris, France. Institut National de la Santé et de la Recherche Médicale-EFS ethical committee permission (N°12/EFS/079) was obtained for the use of buffy coats of healthy donors. Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coats by density gradient centrifugation using Ficoll-paque PREMIUM (GE healthcare, France). CD14<sup>+</sup> monocytes were isolated from PBMCs by using CD14 microbeads (Miltenyi Biotec). Purified monocytes were then cultured for 6 days in RPMI-1640 medium plus 10% fetal calf serum (FCS) containing cytokines GM-CSF (1000 IU/10<sup>6</sup> cells) and IL-4 (500 IU/10<sup>6</sup> cells) (both from Miltenyi Biotec) to obtain DCs<sup>56</sup>. The purity of DCs was >98%. DC-SIGN expression on Mo-DCs was examined by flow cytometry using fluorochrome-conjugated monoclonal antibodies (BD Biosciences) and data were analyzed by FACSDiva™ and FlowJo softwares (Tree Star, USA).

**Isolation of human splenocytes.** The remnant human spleen sections from individuals submitted for pathological diagnosis were obtained from service d'anatomie pathologique, Hôpital Européen Georges Pompidou, Paris, France. Only healthy spleen tissues were used for the research purpose. Since the study did not require additional sampling, an approval from an ethics committee was not required under French law according to the article L.1121-1 of the public health code. The article states that: The research organized and performed on human beings in the development of biological knowledge and medical research are permitted under the conditions laid down in this book and are hereinafter referred to by the term "biomedical research". The article further states that it does not imply under conditions: For research in which all actions are performed and products used in the usual way, without any additional or unusual diagnostic procedure or surveillance.

The spleen sections were collected in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin, and 10% FCS. Single-cell suspension

of splenocytes was obtained by mechanical disaggregation of spleen tissue pieces by using gentleMACS dissociator (Miltenyi Biotec) followed by filtration through 70- $\mu$ m nylon membrane filter (BD Biosciences). Splenocytes were then subjected to Ficoll-Paque PREMIUM density gradient centrifugation to obtain mononuclear cells. DC-SIGN expression on the splenocytes was investigated by flow cytometry using fluorochrome-conjugated monoclonal antibodies and data were analyzed by FACSDiva™ and FlowJo softwares.

**Stimulation of cells.** Mo-DCs ( $0.5 \times 10^6$ /ml) were cultured in RPMI 1640-10% FCS containing GM-CSF and IL-4 in a 12-well plate. The cells were then exposed to IVIg (25 mg/ml) for 48 hours to analyze the effect of IVIg on IL-33 production under non-inflammatory conditions. In parallel, Mo-DCs were stimulated with either TLR4 ligand lipopolysaccharide (100 ng/ml/0.5  $\times 10^6$  cells) (Sigma-Aldrich, France) or inflammatory cytokine cocktail (10 ng/ml each of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , all from ImmunoTools, Germany)<sup>57</sup>. After four hours, IVIg was added and cultures were maintained for 48 hours to analyze the effect of IVIg on IL-33 production under inflammatory conditions.

Similarly, splenocytes ( $0.5 \times 10^6$ /ml) were cultured in RPMI 1640-10% FCS for 48 hours either alone or with IVIg. In addition, splenocytes were also stimulated with inflammatory cytokine cocktail and IVIg was added to the cultures after four hours. The cultures were maintained for 48 hours.

**Quantification of cytokines.** IL-33 in the plasma samples of the patients and in cell-free culture supernatants was quantified by ELISA (R&D systems, France). IL-4 in the plasma was also measured by ELISA (R&D systems).

**Statistical analysis.** Data was analyzed using Prism 5 software (GraphPad software). Two-tailed Student's t-test was used to determine the statistical significance of the data. Values of  $P < 0.05$  were considered as statistically significant.

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## Acknowledgments

This study was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie, Université Paris Descartes and European Community's Seventh Framework Programme [FP7/2007–2013] under grant agreement HEALTH-2010.2.4.5-2 ALLFUN. We also thank Laboratoire Français du Fractionnement et des Biotechnologies, France for the support.

## Author contributions

J.B. designed the research, M.S., C.S., P.H., M.S.M., E.S.-V., L.G. & M.L. performed the experiments, M.S., P.H., M.S.M., S.V.K. & J.B. analyzed the data, Y.S., L.M. & O.B. provided blood samples of the patients, P.B. provided the spleen tissues, J.B. wrote the paper and all authors reviewed and approved the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Sharma, M. *et al.* Intravenous immunoglobulin-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients. *Sci. Rep.* **4**, 5672; DOI:10.1038/srep05672 (2014).



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ARTICLE

Received 5 Nov 2013 | Accepted 9 May 2014 | Published 9 Jun 2014

DOI: 10.1038/ncomms5092

# Human B cells induce dendritic cell maturation and favour Th2 polarization by inducing OX-40 ligand

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Dendritic cells (DCs) play a critical role in immune homeostasis by regulating the functions of various immune cells, including T and B cells. Notably, DCs also undergo education on reciprocal signalling by these immune cells and environmental factors. Various reports demonstrated that B cells have profound regulatory functions, although only few reports have explored the regulation of human DCs by B cells. Here we demonstrate that activated but not resting B cells induce maturation of DCs with distinct features to polarize Th2 cells that secrete interleukin (IL)-5, IL-4 and IL-13. B-cell-induced maturation of DCs is contact dependent and implicates signalling of B-cell activation molecules CD69, B-cell-activating factor receptor, and transmembrane activator and calcium-modulating cyclophilin ligand interactor. Mechanistically, differentiation of Th2 cells by B-cell-matured DCs is dependent on OX-40 ligand. Collectively, our results suggest that B cells have the ability to control their own effector functions by enhancing the ability of human DCs to mediate Th2 differentiation.

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**D**endritic cells (DCs) are professional antigen-presenting cells (APCs) and sentinels of the immune system. They perform a prime role in initiating and controlling antigen-specific immune response<sup>1</sup>. These functions of DCs are enabled by their ability to sense and respond to stimuli from their surroundings and to interact with various cells. To perform these functions, DCs transform from an immature state, where they are superior in sensing (antigenic) stimuli, to a mature state, where they are capable of relaying the antigen signatures to adaptive immune cells to induce antigen-specific immune response<sup>1</sup>. DC maturation is characterized by changes in the expression of antigen-presenting molecule HLA-DR, co-stimulatory molecules and cytokine secretion, which influence the outcome of DC interaction with T and B cells<sup>2</sup>. DCs also produce an array of chemokines based on their maturation status to regulate the trafficking of immune cells.

In fact, DCs engage in a cross-talk with different immune cells, including T cells, natural killer (NK) cells and B cells. Reciprocal signalling by these cells can regulate the DC maturation and functions. Accordingly, several studies have shown that T and B cells, innate lymphocytes and neutrophils could influence the quality of immune responses elicited by DCs<sup>1,3–11</sup>. Activated innate lymphocytes and neutrophils induce maturation of DCs with T helper 1 (Th1) polarizing features<sup>8–11</sup>. However, education of DCs by T cells reliant on their subsets: regulatory T cells (Tregs) induce tolerogenic feature on DCs, whereas naive and effector memory T cells induce DC maturation with potent T-cell stimulatory capacity<sup>4–7</sup>.

B cells are best known for antibody production. Of note, various reports clearly demonstrated that B cells have profound regulatory functions<sup>3,12–17</sup>. However, only few reports have explored the regulation of DC functions by B cells. Murine models have suggested that B cells might favour the induction of non-polarized immune responses by regulating the functions of DCs<sup>18</sup>. Further, a recent study demonstrated that human B cells that receive signalling via CD40 and Toll-like receptor 9 (TLR9) gain the capacity to restrain the maturation and functions of human DCs<sup>19</sup>, although our recent study suggests that regulation of human DCs by B cells depends on the signals they receive<sup>20</sup>. In addition, B cells enhance the production of type I interferon (IFN) by plasmacytoid DCs stimulated with RNA-containing immune complexes<sup>21</sup>.

Thus far, only few reports have explored the regulation of human DCs by B cells. Therefore, in the present study we investigated whether B cells could positively regulate human DC maturation and function. We demonstrate that on B-cell receptor (BCR) or CD40-mediated activation, human B cells induce maturation of DCs characterized by enhanced expression of HLA-DR and co-stimulatory molecules CD80, CD86 and CD40. For B cells to exert these effects on DCs, direct cellular contact mediated through molecules associated with B-cell activation such as CD69, B cell-activating factor receptor (BAFF-R) and transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) are essential. Further, activated B cells also induce increased secretion of cytokines and selectively modulate the chemokine production of DCs. These B-cell-matured DCs exhibit enhanced CD4<sup>+</sup> T cell (Th) stimulatory capacity with unique features to promote Th2 response without affecting other effector Th cell subsets and Tregs.

## Results

**BCR-activated human B cells induce maturation of DCs.** We first investigated the effect of B cells on phenotype of DCs. Freshly isolated circulating CD19<sup>+</sup> B cells, referred as 'resting B cells', were cultured with immature DCs for 48 h (Fig. 1a).

We found that resting B cells do not significantly modify the phenotype of DCs (DC<sub>Rest-B</sub>) and the expression of various DC molecules was similar to that of control DCs (DC<sub>ctrl</sub>) (Fig. 1b–d). These results indicate that in resting phase, B cells do not provide signals to DCs to undergo maturation. Interestingly, when BCR signalling was provided by F(ab')<sub>2</sub> fragments of anti-human IgM antibodies in the DC–B cell co-culture (Fig. 1a), the activated B cells induced maturation of DCs (DC<sub>BCR-B</sub>). Thus, DCs showed significantly enhanced expression of co-stimulatory molecules CD80, CD86 and CD40, antigen-presenting molecule HLA-DR and terminal maturation marker CD83 (Fig. 1b,c). Consensus with the DC maturation, the expression of antigen uptake receptor CD206 and lipid antigen-presenting molecule CD1a were decreased, whereas the levels of integrin receptor CD11c and C-type lectin receptor DC-SIGN were increased on DC<sub>BCR-B</sub> (Fig. 1d). Similar results were observed with allogenic as well as autologous B cell–DC co-culture, suggesting that the changes in DC phenotype are not due to allogeneic reaction.

We confirmed that the maturation of DCs was indeed due to signalling by BCR-activated B cells and not due to nonspecific stimulation of DCs by F(ab')<sub>2</sub> fragments of anti-human IgM antibodies used for the BCR activation of B cells. The phenotype of DCs was unaltered when cultured with F(ab')<sub>2</sub> fragments of anti-human IgM alone, in the absence of B cells (Supplementary Fig. 1). Further, induction of maturation of DCs by BCR-activated B cells varied directly with the DC:B cell ratio (Fig. 1e). Together, these data demonstrate that BCR-mediated activation license human B cells to induce maturation of DCs.

## Induction of DC maturation by B cells is contact dependent.

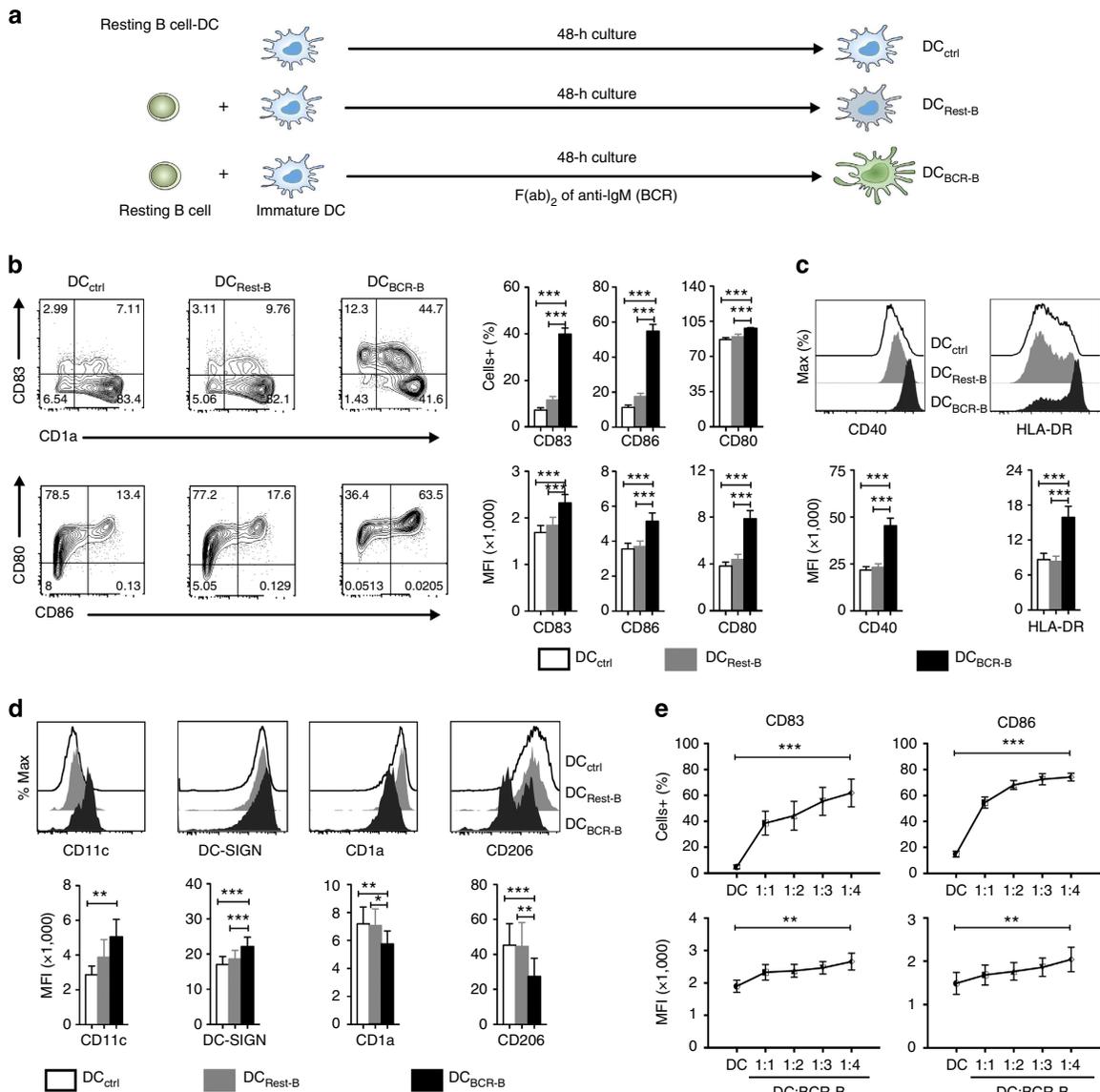
B cells could regulate immune responses both by direct cellular interactions and by secreting various soluble mediators<sup>3,12,19</sup>. Therefore, we explored the mode of interaction involved in the induction of DC maturation by B cells. The B cells were pre-activated with BCR alone (DC<sub>pBCR-B</sub>) or with BCR + CD40-stimulation (DC<sub>pBCR+CD40-B</sub>) and immature DCs were co-cultured with these B cells either in direct contact or kept separated in transwells (Fig. 2a). In parallel, DCs were also stimulated with supernatant from activated B cells.

We found that DCs co-cultured in direct contact with activated B cells exhibit phenotypic changes suggestive of maturation, including increased expression of CD83, CD80, CD86, CD40 and HLA-DR (Fig. 2b,c, panel 'Contact'). Interestingly, BCR-mediated activation alone appears to be sufficient for B cells to gain capacity to induce maturation of DCs (DC<sub>pBCR-B</sub>) as B cells that received additional stimulation via CD40 also induced similar level of maturation of DCs (DC<sub>pBCR+CD40-B</sub>) (Fig. 2b,c). Furthermore, B cells that received activation signalling via CD40 alone also induced maturation of DCs (Supplementary Fig. 2). Thus, BCR or CD40 signalling enables B cells to induce DC maturation.

In contrast to direct contact, when pre-activated B cells were separated from DCs in transwell plates, DC maturation-associated molecules were not upregulated and DCs retained the phenotype similar to control cells (DC<sub>ctrl</sub>) (Fig. 2b,c, panel 'Trans'). Even soluble mediators present in the supernatants of activated B cells did not promote maturation of DCs (Fig. 2b,c; panel 'Supernt'). Taken together, these results indicated that direct contact with activated B cells is indispensable and sufficient for the induction of maturation of DCs. Of interest, BCR-pre-activated B cells that were paraformaldehyde fixed also induced maturation of DCs, thus suggesting a role for B-cell surface molecules in the process (Supplementary Fig. 3).

## B-cell activation-associated molecules mediate DC maturation.

As activation of B cells and contact-dependent interaction

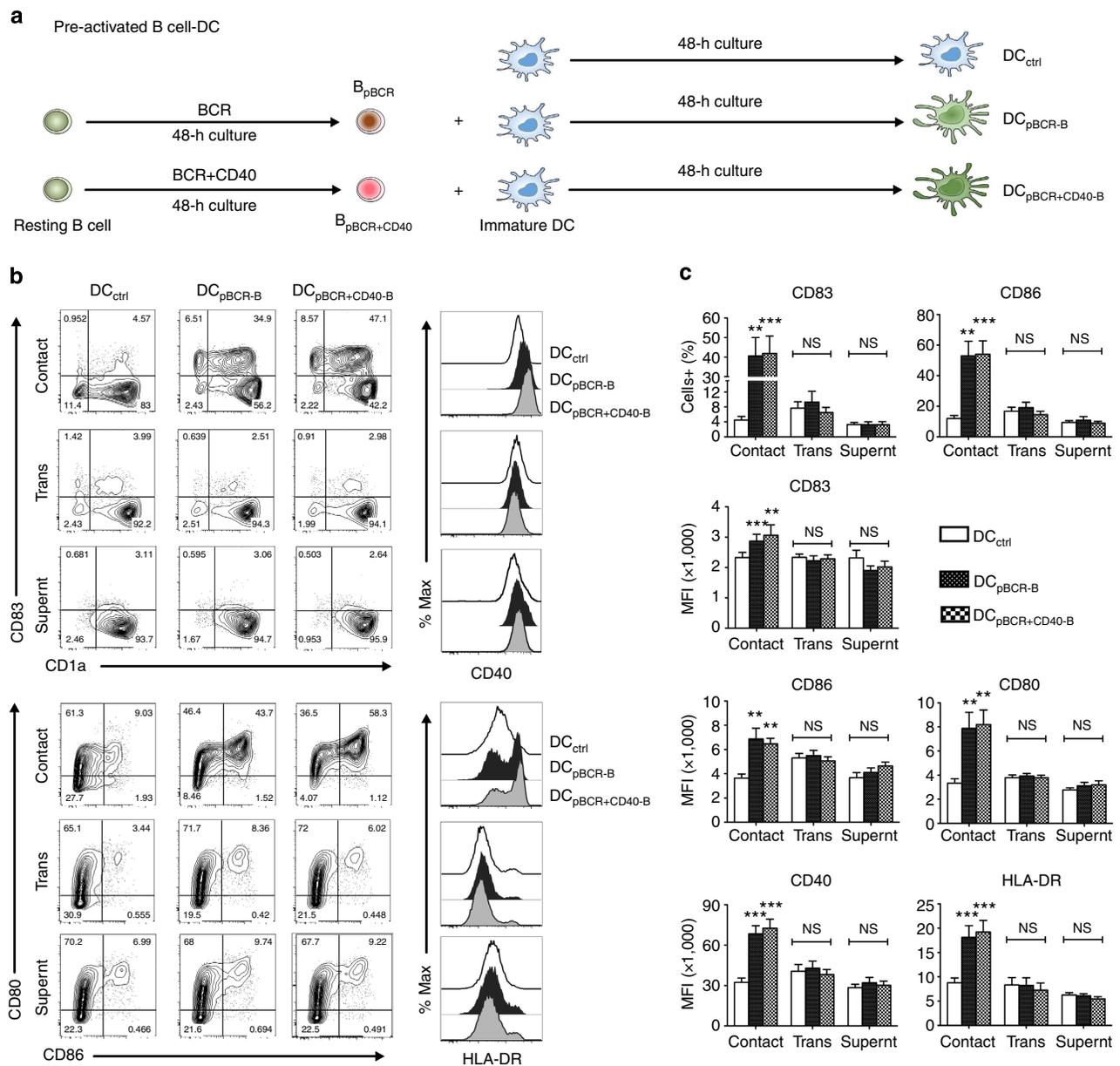


**Figure 1 | BCR-activated B cells induce maturation of DCs.** (a) Experimental design. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone ( $DC_{ctrl}$ ), or co-cultured at 1:1 ratio with  $CD19^+$  B cells that were either in resting phase ( $DC_{Rest-B}$ ) or directly activated in DC-B cell co-culture via BCR stimuli ( $DC_{BCR-B}$ ). Phenotypic analysis of CD20-negative cells was done by flow cytometry. (b) BCR-activated B cells enhance the expression (% positive cells and mean fluorescence intensity, MFI) of DC activation markers and co-stimulatory molecules. (c) BCR-activated B cells upregulate expression of HLA-DR and CD40 on DCs. (d) Modulation of expression of CD11c, DC-SIGN, CD1a and CD206 on DCs by BCR-activated B cells. Representative plot and mean  $\pm$  s.e.m. of data from 10 to 24 donors. (e) Immature DCs were co-cultured with increasing number of B cells in the presence of BCR stimuli for 48 h and expression (% positive cells and MFI) of CD83 and CD86 was analysed on CD20-negative cells. Mean  $\pm$  s.e.m. of data from four donors. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  by one-way analysis of variance test.

between DCs and B cells were necessary for the maturation of DCs, we aimed at identifying the activation-associated B-cell surface molecules implicated in this process. We found that in addition to upregulation of classical B-cell activation-associated molecules, such as HLA-DR, CD25, CD38, CD40, CD80 and CD86, B-cell stimulation also led to significantly enhanced expression of CD69, BAFF-R and TACI (Fig. 3a,b). We found that BCR ( $B_{BCR}$ ) or BCR + CD40 ( $B_{BCR+CD40}$ ) signalling was superior to CD40 signalling alone in their ability induce the expression of B-cell activation-associated molecules (Fig. 3a,b). As CD69, BAFF-R and TACI could interact with corresponding ligands on DCs, we explored the role of these B-cell surface

molecules in the process of DC maturation by employing blocking antibodies.

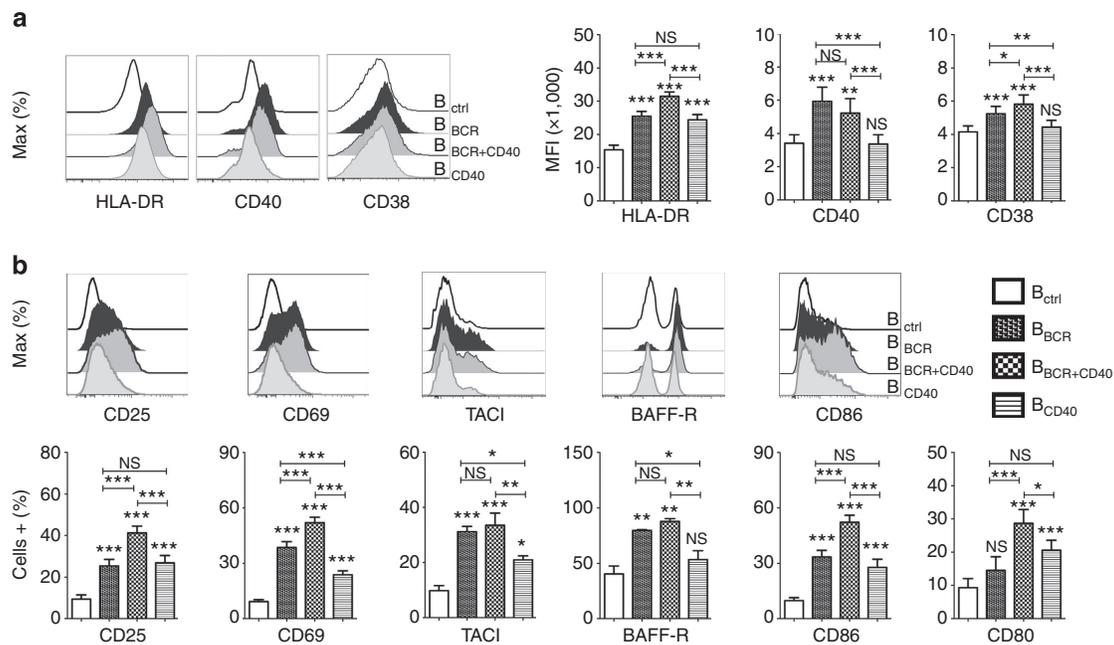
Interestingly, on blocking TACI, BAFF-R or CD69 individually lead to only minor inhibition of DC maturation. However, blocking all three receptors simultaneously lead to significant inhibition of DC maturation as analysed by the phenotype (Fig. 4). Of note, adhesion molecules CD54 and CD11a/CD18 were found to be not involved in the process. We also found that the expression of CD69 on B cells implicates BCR or CD40 signals received by the B cells but not feedback signals by BAFF as treatment of B cells with BAFF led to only minor changes in the expression of CD69 on B cells.



**Figure 2 | Cellular contact is required and sufficient for the induction of maturation of DCs by activated B cells.** (a) Pre-activated B cell-DC experimental design. Immature DCs were cultured in medium containing granulocyte macrophage colony-stimulating factor and IL-4 for 48 h either alone (DC<sub>ctrl</sub>) or co-cultured at 1:1 ratio with CD19<sup>+</sup> B cells that were pre-activated by BCR (DC<sub>pBCR-B</sub>) or BCR + CD40 (DC<sub>pBCR+CD40-B</sub>) stimulation. (b,c) DC-B cell co-culture was either done in 96 U-bottomed wells to allow direct contact between DCs and B cells (Contact) or in transwell plate to separate the B cells from DCs (Trans). Immature DCs were also cultured in the supernatants from activated B cells (Supernt). Phenotypic analysis (% positive cells and mean fluorescence intensity, MFI) of DCs that were gated negative for CD20. Representative plot and mean  $\pm$  s.e.m. of data from 7 to 11 donors. (Contact,  $n = 11$ ; Transwell,  $n = 7$ ; Supernatant,  $n = 8$ ). \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; NS, not significant by one-way analysis of variance test.

**Modulation of DC cytokines and chemokines by activated B cells.** During the process of maturation, in addition to up-regulation of expression of surface markers, DCs also secrete increased amount of various cytokines and chemokines that play a critical role in creating inflammation, polarization of T cells and migration of diverse immune cells. Analysis of supernatants from DC-B cell co-cultures showed that in the presence of resting B cells (DC<sub>Rest-B</sub>) only marginal changes in the cytokine profile were observed (Fig. 5a). When B cells were activated directly in the DC-B cell co-culture via BCR signalling (DC<sub>BCR-B</sub>), B cells stimulated DCs to produce significant amount of IL-6, tumour necrosis factor (TNF)- $\alpha$  and IL-10 (Fig. 5a). Similarly, co-culture of DCs in direct

contact with B cells that were pre-activated with either BCR alone (DC<sub>pBCR-B</sub>) or BCR + CD40 (DC<sub>pBCR+CD40-B</sub>) significantly enhanced the production of above cytokines (Fig. 5b). However, DC co-cultured with pre-activated B cells in transwell culture system did not produce increased amount of cytokines. As the above cytokines in the co-culture could be derived from DCs as well as B cells, we wanted to confirm that increased secretion of cytokines in DC-B cell co-culture was mainly because of DCs. In fact, intracellular staining showed that activated B cells induced significantly increased frequency of IL-6- and TNF- $\alpha$ -producing DCs (Fig. 5c), suggesting that increased cytokine levels in DC-B cell co-culture were mainly contributed from DCs.



**Figure 3 | Phenotypic analysis of B cells activated with various stimuli.** CD19<sup>+</sup> B cells were either cultured alone ( $B_{ctrl}$ ) or in the presence of BCR ( $B_{BCR}$ ) or agonistic CD40 antibody ( $B_{CD40}$ ) alone, or BCR + CD40 ( $B_{BCR+CD40}$ ) for 48 h. **(a,b)** Expression of surface activation markers on B cells as analysed by flow cytometry. Representative plot and mean  $\pm$  s.e.m. of data from five to ten donors. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; NS, not significant. Statistical significance as determined by one-way analysis of variance test.

Next, we analysed the supernatant of B cell–DC co-culture for various chemokines that could influence the trafficking of immune cells. Similar to cytokine profiles, irrespective of B-cell activation method, that is, B cells activated directly in the DC:B cell co-cultures via BCR signalling ( $DC_{BCR-B}$ ) (Fig. 5d) or pre-activated with BCR ( $DC_{pBCR-B}$ ), or with BCR + CD40 ( $DC_{pBCR+CD40-B}$ ) before co-culture with DCs (Fig. 5e), stimulated DCs to produce significantly increased amounts of CCL17 and CCL22 (ligands for CCR4), CCL3 and CCL4 (ligands for CCR5), and CXCL8 (IL-8, ligand for CXCR1 and CXCR2). In contrast, CXCL16 (ligand of CXCR6), and CXCL9 and CXCL10 (ligands for CXCR3), were not significantly altered (Fig. 5d,e).

To explore the contribution of DCs to the chemokines in the B cell–DC co-culture, we analysed the supernatants of activated B cells. On BCR ( $B_{BCR}$ ) or BCR + CD40 ( $B_{BCR+CD40}$ )-mediated activation, B cells did not secrete CCL17, CCL22 and CXCL16, indicating that these chemokines in DC–B cell co-cultures were contributed mainly by DCs. However, the secretion of CCL3, CCL4, CXCL9 and CXCL10 by activated B cells was either on par with those levels observed in DC–B cell co-cultures or even higher, suggesting that these chemokines in DC–B cell co-cultures were contributed mainly by B cells rather than DCs (Supplementary Fig. 4a). Furthermore, analysis of intracellular expression of chemokines in DCs revealed that activated B cells enhanced the frequency of CXCL8-producing DCs, but not CCL4 (Supplementary Fig. 4b). Together, these findings provide a pointer towards selective induction of DC chemokines by activated B cells.

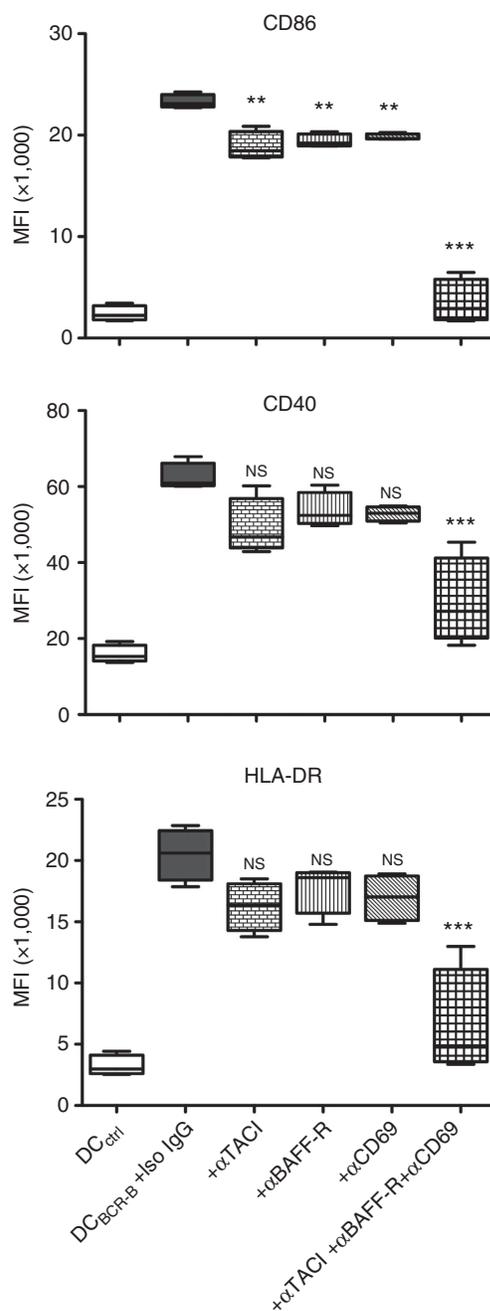
**T-cell stimulation and polarization by B-cell-matured DCs.** DCs matured under the influence of B cells were evaluated for T-cell stimulatory capacity by co-culturing B-cell-depleted DCs with CD4<sup>+</sup> T cells (Th cells) (Fig. 6a). Corroborating the DC phenotype and cytokine profile, resting B cells did not alter the capacity of DCs ( $DC_{Rest-B}$ ) to induce T-cell proliferation and was similar to control DCs ( $DC_{ctrl}$ ). On the contrary, DCs that were

‘educated’ by activated B cells ( $DC_{BCR-B}$ ) stimulated significant proliferation of T cells (Fig. 6b) and was associated with significant production of T-cell-derived cytokine IL-2 (Fig. 6c). Further, our results also indicated that DCs that received maturation signals from B cells pre-activated with either BCR alone ( $DC_{pBCR-B}$ ) or with BCR + CD40-stimulation ( $DC_{pBCR+CD40-B}$ ) were similar in their ability to stimulate T-cell proliferation and to induce IL-2 in proliferating T cells (Supplementary Fig. 5a). On the contrary, DCs cultured either in transwell plates (Supplementary Fig. 5b) or with supernatants of activated B cells (Supplementary Fig. 5c) did not promote T-cell proliferation and IL-2 secretion, thus further confirming the indispensability of cellular contact between DCs–B cells for the functional maturation of DC.

To further understand the influence of B-cell-matured DCs on CD4<sup>+</sup> T-cell responses, we measured the levels of various Th cell cytokines in the DC–T cell supernatants. Interestingly,  $DC_{BCR-B}$  skewed Th-cell responses towards Th2 as indicated by significant secretion of Th2 cell cytokines, including IL-4, IL-5 and IL-13 (Fig. 6d). In addition, modest increase in TNF- $\alpha$  and IL-22 was also observed (Fig. 6e). However, IFN- $\gamma$ , signature cytokine of Th1 and IL-17, signature cytokine of Th17, and also IL-21 and IL-10 were not altered (Fig. 6f).

### B-cell-matured DCs selectively induce Th2 differentiation.

Next we investigated the ability of B-cell-matured DCs to modulate the differentiation of naive Th cells. We found that DCs that received signals from BCR-activated B cells ( $DC_{BCR-B}$ ) significantly increased the frequencies of Ki-67<sup>+</sup> and IL-2<sup>+</sup> T cells ( $T_{BCR-B-DC}$ ) (Fig. 7a,b), indicating enhanced proliferation of naive T cells. However, frequencies of Ki-67<sup>+</sup> and IL-2<sup>+</sup> T cells were not significantly altered when naive Th cells were co-cultured with DCs that received signals from resting B cells ( $T_{Rest-B-DC}$ ). Moreover, naive T cells cultured with  $DC_{BCR-B}$  significantly downregulated the expression of CD27 and CCR7 compared with T cells cultured with  $DC_{ctrl}$  or  $DC_{Rest-B}$  (Fig. 7c,d). These results



**Figure 4 | Several surface molecules on activated B cells are involved in contact-dependent induction of maturation of DCs.** Immature DCs were cultured in medium containing granulocyte macrophage colony-stimulating factor and IL-4 for 48 h either alone ( $DC_{ctrl}$ ), or co-cultured at the ratio of 1:1 with  $CD19^+$  B cells in the presence of BCR stimuli. Co-culture was done either in the presence of isotype control mouse IgG ( $DC_{BCR-B} + Iso\ IgG$ ) or blocking antibodies to TACI ( $+ \alpha TACI$ ), BAFF-R ( $+ \alpha BAFF-R$ ), CD69 ( $+ \alpha CD69$ ) alone or together ( $+ \alpha TACI + \alpha BAFF-R + \alpha CD69$ ). Flow cytometric analysis of phenotype (mean fluorescence intensity, MFI) of CD20-negative DC population was performed. Mean  $\pm$  s.e.m. of data from four donors.  $**P < 0.01$ ;  $***P < 0.001$ ; NS, not significant by one-way analysis of variance test.

suggested that B-cell-matured DCs induce proliferation and differentiation of effector T cells.

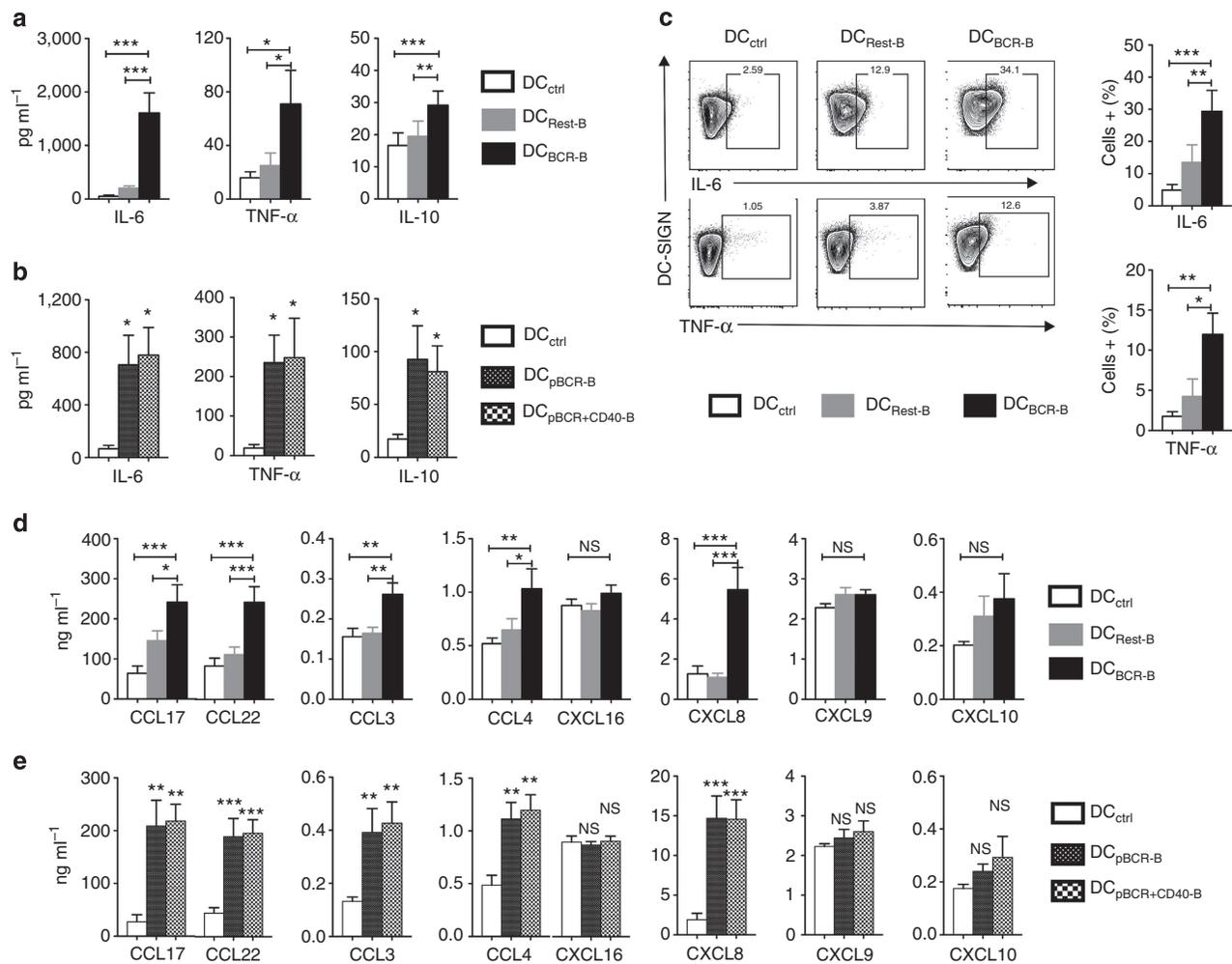
Of note,  $CCR7^- CD27^-$  T cells were previously shown to contain mainly the IL-4-producing T cells<sup>22</sup>. In line with this

report, we further found that  $DC_{BCR-B}$  significantly increased the frequency of  $CCR4^+$  T cells but did not alter the expression of CXCR3, CXCR5 and CCR6 on T cells (Fig. 7c,d). Of particular relevance, CCR4 has been shown to be preferentially expressed on Th2 cells and Tregs<sup>23</sup>. Interestingly, we found that  $DC_{BCR-B}$  significantly increased the frequency of IL-4-expressing cells but not FoxP3<sup>+</sup> cells (Fig. 7a,b). Thus, our findings suggest that B-cell-matured DCs selectively induce differentiation of Th cells towards Th2 phenotype and not Tregs. However, the frequency of IFN- $\gamma$ -expressing Th1 cells and IL-17-secreting Th17 cells was unaltered by  $DC_{BCR-B}$  (Fig. 7a,b).

Further, analysis of the supernatants for T-cell cytokines revealed that  $DC_{BCR-B}$  significantly increased the secretion of IL-2, which supports the proliferating T cells (Fig. 7e). Interestingly, these T cells ( $T_{BCR-B-DC}$ ) secreted significantly higher amount of Th2 cytokines such as IL-4, IL-5 and IL-13 (Fig. 7e). However, the secretion of IFN- $\gamma$  and IL-17 by T cells was not significantly altered by  $DC_{BCR-B}$  (Fig. 7e). Finally, we examined the expression of Th cell subset-specific transcription factors to substantiate the surface phenotype and cytokine profile data of T cells.  $DC_{BCR-B}$  significantly enhanced the expression GATA3, the master regulator of Th2 cells (Fig. 7f)<sup>24</sup>, while the expression of T-bet (Th1), RORC (Th17) and Foxp3 (Tregs) was not altered by  $DC_{BCR-B}$  (Fig. 7a,b,f). Of note, DCs activated by B cells pre-stimulated with BCR ( $DC_{pBCR-B}$ ) or CD40 alone ( $DC_{pCD40-B}$ ), or BCR + CD40 ( $DC_{pBCR+CD40-B}$ ), were also capable of inducing naive Th-cell activation, proliferation and differentiation to Th2 phenotype to a similar extent (Supplementary Fig. 6). Taken together, our results demonstrate that DCs that receive signals from activated B cells selectively induce differentiation of Th2 cells from naive T cells.

**Th2 polarization by B-cell-matured DCs is OX-40 ligand-dependent.** We explored the mechanism of induction of Th2 cells by B-cell-matured DCs. We observed that activated B cells did not induce Th1-triggering molecules in DCs such as IL-12 and CD70 (Fig. 8a,c), which is in consensus with the unaltered IFN- $\gamma$ -expressing cells and IFN- $\gamma$  secretion by T cells that were stimulated by B-cell-matured DCs. Further, B cells did not stimulate the production of IL-33 in DCs, a cytokine that was shown to amplify both Th1- and Th2-type responses in humans (Fig. 8b)<sup>25</sup>.

As DCs did not produce Th2-promoting cytokines, we next investigated the expression of Th2-inducing surface molecules on DCs. Interestingly, we found that activated B cells, but not resting B cells, significantly upregulated the expression of OX-40 ligand (OX-40L) on DCs (Fig. 8d). Thus, upregulation of OX-40L in the absence of IL-12 and CD70 expression on B-cell-matured DCs suggested that its interaction with OX-40 on T cells is responsible for driving Th2 polarization. Of particular relevance, epithelial cell-derived thymic stromal lymphopoietin (TSLP) was also shown to upregulate the expression of OX-40L on DCs and is implicated in Th2 responses during allergy reactions<sup>26</sup>. Hence, we compared the phenotype and T-cell-stimulating abilities of TSLP-treated DCs ( $DC_{TSLP}$ ) and B-cell-matured DCs ( $DC_{pBCR-B}$ ). Surprisingly, with the exception of CD86, TSLP did not significantly upregulate the activation-associated molecules on DCs and  $DC_{TSLP}$  failed to induce significant T-cell proliferation, as compared with  $DC_{pBCR-B}$  (Supplementary Fig. 7a,b,d). Interestingly, although the expression of GATA3 was enhanced only by  $DC_{pBCR-B}$ , both  $DC_{TSLP}$  as well as  $DC_{pBCR-B}$  enhanced the production of Th2 cytokines, IL-5 and IL-13 to a similar extent, without modifying IFN- $\gamma$  secretion (Supplementary Fig. 7e,f). Consistent with this observation, induction of OX-40L expression on DCs by BCR- or CD40-activated B cells was similar to that of TSLP signalling (Supplementary Fig. 7c). These findings further



**Figure 5 | Effect of activated B cells on the secretion of cytokines and chemokines by DCs.** Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC<sub>ctrl</sub>), or co-cultured at 1:1 ratio with CD19<sup>+</sup> B cells that were either in resting phase (DC<sub>Rest-B</sub>) or directly activated in DC-B cell co-culture via BCR stimuli (DC<sub>BCR-B</sub>). Alternatively, DCs were also co-cultured with CD19<sup>+</sup> B cells that were pre-activated by BCR (DC<sub>pBCR-B</sub>) or BCR + CD40 (DC<sub>pBCR+CD40-B</sub>) stimulation for 48 h. **(a,b)** Supernatants of co-cultures were analysed for the amounts (pg ml<sup>-1</sup>) of cytokines. Mean ± s.e.m. of data from ten donors. **(c)** CD20-negative DCs from B cell-DC co-cultures were analysed for the intracellular expression of IL-6 and TNF-α by flow cytometry. Representative plot and mean ± s.e.m. of data from four donors. **(d,e)** Amount of chemokines in the culture supernatants of DC-B cell co-cultures. Mean ± s.e.m. of data from ten donors. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; NS, not significant by one-way analysis of variance test.

support that B-cell-matured DC-induced Th2 polarization involves OX-40L.

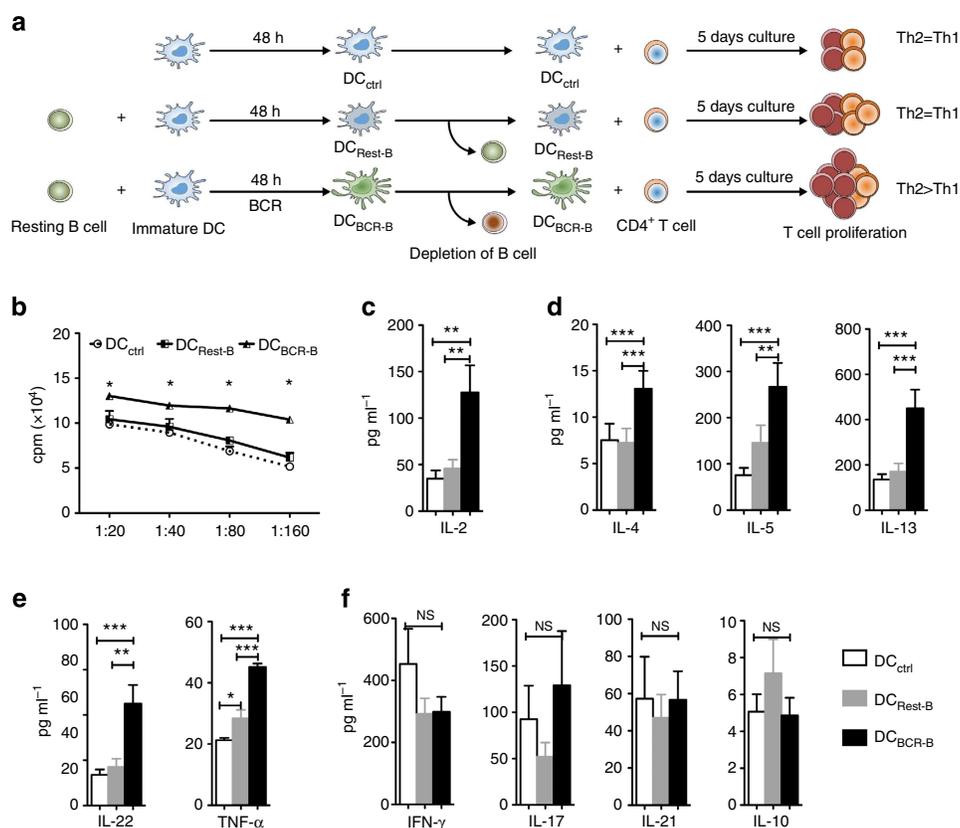
To confirm this, blocking antibodies to OX-40L were employed in the co-culture of naive T cells and DC<sub>BCR-B</sub> (Fig. 8e). Following 6 days of culture, the frequencies of Ki-67<sup>+</sup> and IL-2<sup>+</sup> T cells in the presence of anti-OX-40L antibodies were similar to isotype antibodies (Fig. 8f,g), suggesting that blocking OX-40L did not interfere with DC-induced proliferation and survival of naive T cells. Although blocking of OX-40L did not inhibit the frequency of IL-4-expressing T cells (Fig. 8f,g), there was significant decrease in the amount of secretion of Th2 cytokines, IL-4, IL-5 and IL-13 (Fig. 8h). Together, our results demonstrate that Th2 differentiation by B-cell-matured DCs is dependent at least in part on OX-40 ligand.

**Th2 cells induced by B-cell-matured DCs promote IgE production.** To further evaluate the physiological role of Th2 polarization induced by B-cell-matured DCs, we co-cultured the differentiated T cells with freshly isolated B cells and determined

their ability to induce IgE production (Supplementary Fig. 8a). Preliminary results revealed that Th2 cells induced by B-cell-matured DCs are capable of stimulating B cells to produce IgE, which was similar to that of T cells activated by TSLP-DC (Supplementary Fig. 8b). However, the levels of IgE induced by these Th2 cells were low and variable, and more studies with additional donors are required to provide fully conclusive results.

## Discussion

Of much importance in recent advances in B-cell biology, IL-10-secreting regulatory B cells are claimed to restrain the inflammatory responses by inhibiting proinflammatory cytokines, Th1/Th17 cell development and by facilitating Treg differentiation<sup>12,15,27,28</sup>. In addition, B-cell gene therapy approach has been exploited for the induction of antigen-specific immune tolerance<sup>29</sup>. However, pathogenic role of B cells by the way of T-cell stimulation or via autoantibodies is well documented in autoimmune and inflammatory diseases<sup>30–33</sup>. Thus, B cells exert both effector and regulatory functions in co-ordination with



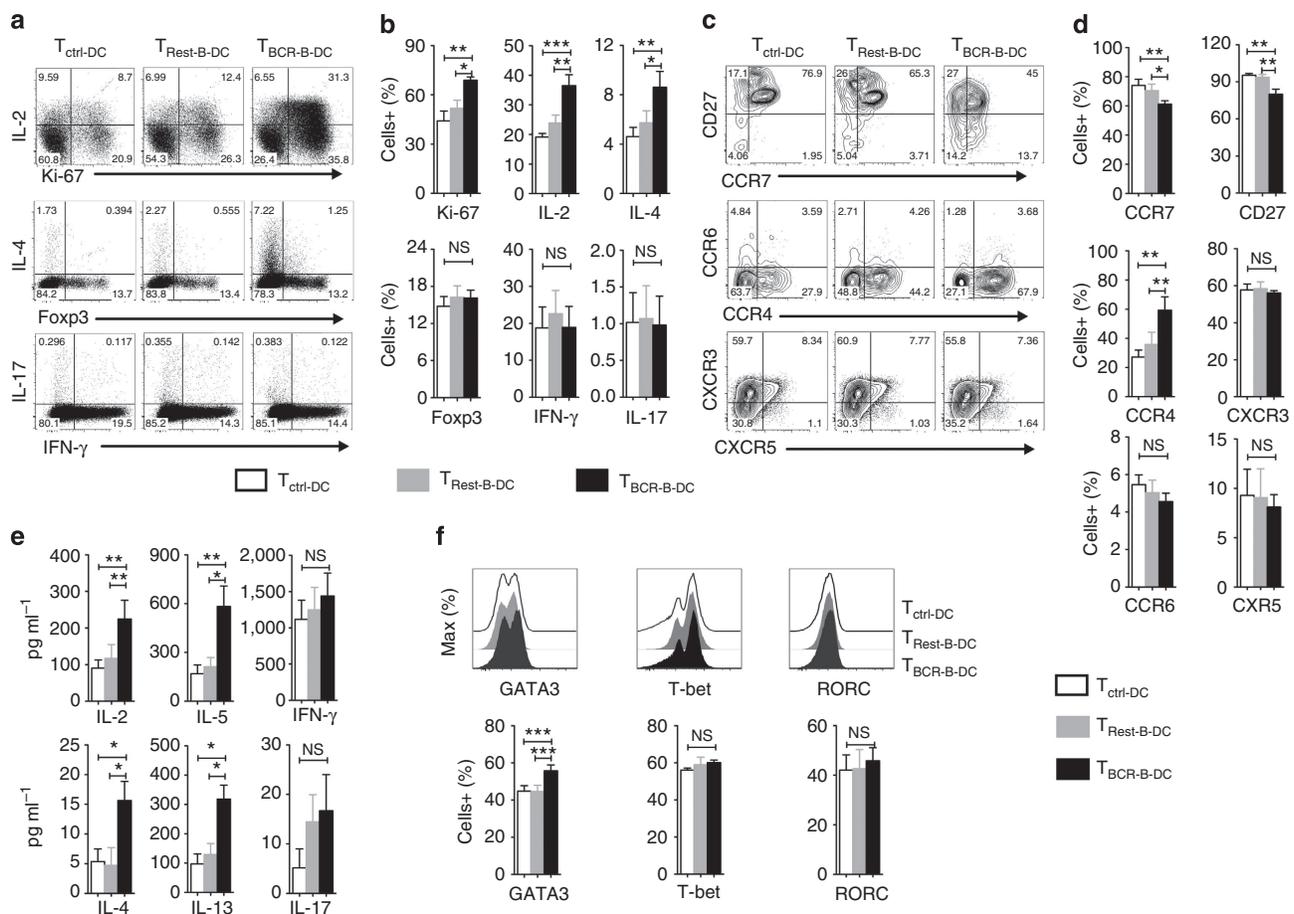
**Figure 6 | B-cell-matured DCs induce proliferation of CD4<sup>+</sup> T cells producing increased amounts of Th2 type cytokines.** (a) Experimental design. Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC<sub>ctrl</sub>) or co-cultured at 1:1 ratio with CD19<sup>+</sup> B cells that were either in resting phase (DC<sub>Rest-B</sub>) or directly activated in DC-B cell co-culture via BCR stimuli (DC<sub>BCR-B</sub>). DCs were purified from B cell-DC co-culture by depleting B cells using CD20 microbeads. CD4<sup>+</sup> T cells were co-cultured with DC at various ratios for 4 days. (b) DC-mediated CD4<sup>+</sup> T-cell proliferation as determined by [<sup>3</sup>H] incorporation assay. Values are presented as counts per minute (cpm). Data are mean ± s.e.m. from quadruplicate wells and representative of five independent experiments. (c–f). Supernatants from DC-T cell co-cultures at 1:20 ratio were analysed for different T-cell cytokines. Mean ± s.e.m. of data from eight to ten donors. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; NS, not significant by one-way analysis of variance test.

T cells and DCs during antigen-specific response. Our results demonstrate that B cells when activated, but not at resting phase, induce maturation of DCs with distinct features to polarize T cells. In consensus with our report, previous studies have also shown that human DC maturation could be induced on interaction with activated immune cells, but not with resting cells, wherein naive and effector T cells, IL-2-activated NK cells, antigen-activated NKT cells and  $\gamma\delta$  T cells, and also TLR-stimulated neutrophils induce maturation of DCs and increase their T-cell stimulatory capacity<sup>1,3,7–10</sup>.

Several reports have demonstrated that B cells have the capacity to regulate the various facets of DC functions both *in vitro* and *in vivo*, to coordinate the process of immune response. B-cell-derived cytokines and chemokines play an important role in the migration of DC subsets. By guiding the expression of CXCL13 on lymphotoxin  $\beta$ -receptor-expressing follicular stromal cells in the spleen, B cell-derived membrane lymphotoxin has been demonstrated to synchronize the migration of a CXCR5<sup>+</sup>CD11c<sup>+</sup> subset of murine DCs for the development of IL-4-producing Th2 cells<sup>34,35</sup>. By binding to CD4, IL-16, a polypeptide cytokine secreted by B cells, could induce migration of circulating human CD4<sup>+</sup>CD11c<sup>+</sup> blood DC subset<sup>36</sup>. As IL-16 could also induce migration of CD4<sup>+</sup> T cells, B cells might mediate cross-talk between DC-CD4<sup>+</sup> T cells–B cells. In addition, via production of antibodies, B cells are also

known to regulate DC maturation, activation and functions by engaging Fc receptors or non-Fc receptors, such as CD40, or C-type lectin receptors and T-cell activation<sup>3,37–43</sup>.

A previous study in B-cell-deficient mice has indicated that B cells might affect the Th1/Th2 cytokine balance by regulating the function of DCs<sup>18</sup>. The authors found that DCs from B-cell-deficient  $\mu$ MT mice that were injected with keyhole limpet haemocyanin emulsified in complete Freund's adjuvant, induced differentiation of CD4<sup>+</sup> T cells that secreted IL-2, IFN- $\gamma$ , IL-5 and IL-10. However, unlike DCs from wild-type mice, they failed to induce IL-4. Mechanistically, as compared with DCs from wild-type mice, DCs from  $\mu$ MT mice produced higher levels of IL-12 when pulsed with keyhole limpet haemocyanin. This report suggested that B-cell-derived IL-10 might regulate the production of IL-12 by DCs and consequently Th1/Th2 cytokine response. Similarly, DCs from B-cell-deficient mice infected with *Candida albicans* and *Aspergillus fumigatus* also produced higher quantities of IL-12 and lower amounts of IL-10 that might favour enhanced Th1 responses observed in these mice<sup>44</sup>. In line with these reports, B-cell-derived IL-10 was found to be critical for favouring Th2 polarization in *Leishmania* infection model in mice by downregulating IL-12 production by DCs<sup>45</sup>. However, in our study and in another report, CD19<sup>+</sup> peripheral human B cells, when activated through BCR alone or with CD40, did not produce significant amounts of IL-10 (ref. 16). These results thus



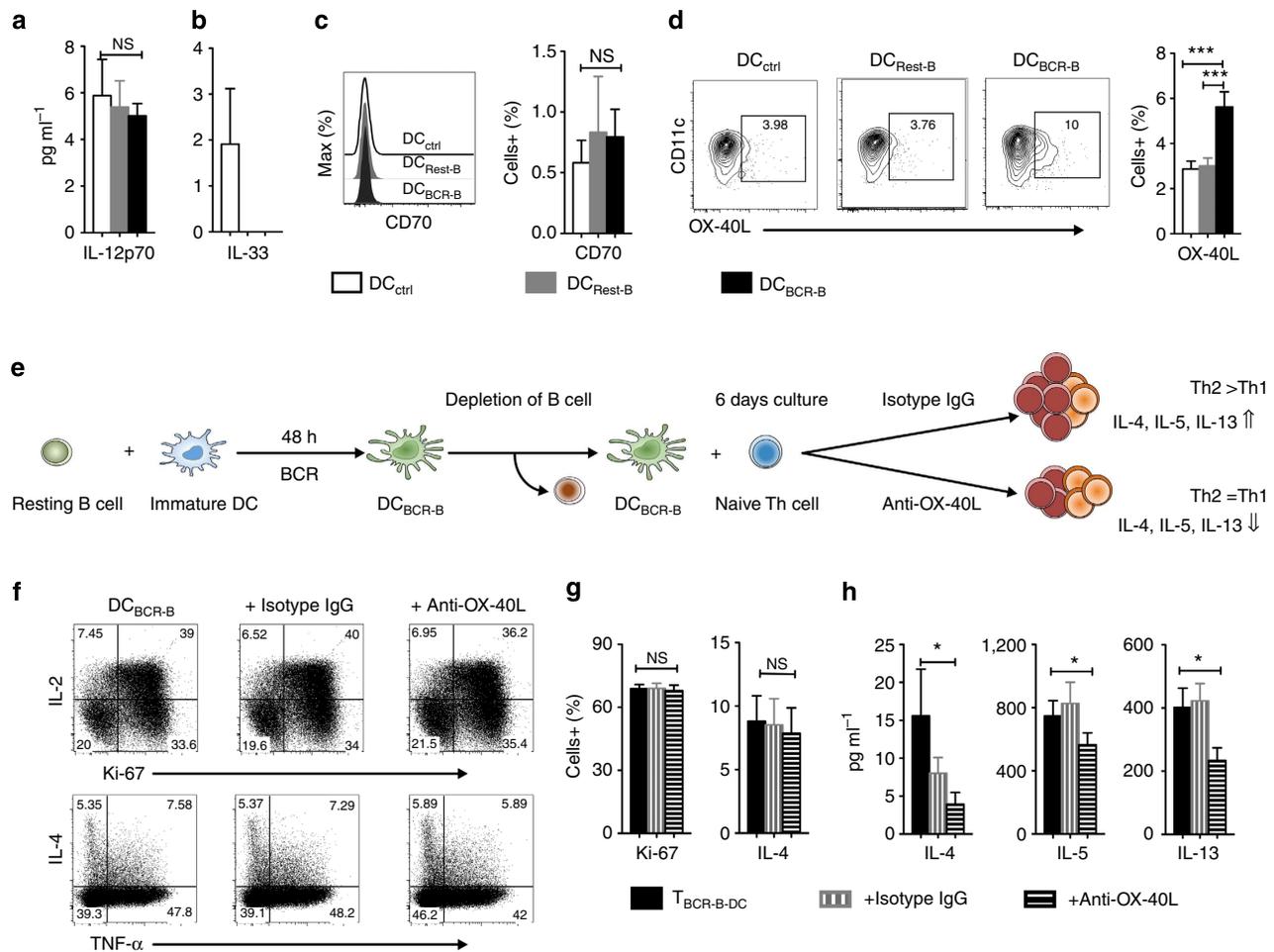
**Figure 7 | B cell-matured DCs favour polarization of naive CD4<sup>+</sup> T cells to Th2 cells.** Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone (DC<sub>ctrl</sub>), or co-cultured at 1:1 ratio with CD19<sup>+</sup> B cells that were either in resting phase (DC<sub>Rest-B</sub>) or directly activated in DC-B cell co-culture via BCR stimuli (DC<sub>BCR-B</sub>). DCs were purified from DC-DC co-culture by depleting B cells. CD4<sup>+</sup>CD45RO<sup>-</sup> naive Th cells were co-cultured with control DCs (T<sub>ctrl-DC</sub>) or activated B-cell-matured DCs (T<sub>Rest-B-DC</sub>) or resting B-cell-stimulated DC (T<sub>BCR-B-DC</sub>) at the ratio of 20:1 for 6 days. **(a,b)** CD3<sup>+</sup> T cells were analysed for the intracellular expression of cytokines and transcription factors by flow cytometry. Representative plot and mean  $\pm$  s.e.m. of data from ten donors. **(c,d)** CD3<sup>+</sup> T cells were analysed for the surface phenotype by flow cytometry. Representative plot and mean  $\pm$  s.e.m. of data from five donors. **(e)** After 6 days, supernatants from DC-T cell co-cultures were analysed for various T-cell cytokines. Mean  $\pm$  s.e.m. of data from eight to ten donors. **(f)** CD3<sup>+</sup> T cells were analysed for intracellular expression of transcription factors by flow cytometry. Representative plot and mean  $\pm$  s.e.m. of data from four donors. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; NS, not significant by one-way analysis of variance test.

indicated that B cells could regulate DC cytokines and T-cell polarization both via cytokine-dependent (similar to IL-10) and -independent manner (as described in this report), depending on the context of immune response or possibly depending on the particular subset of B cells.

Morva *et al.*<sup>19</sup> recently reported that B cells that received activation signalling via combination of TLR9 (CpG) and CD40 restrain the maturation and function of human DCs. It is known that TLR9 signalling in combination with BCR stimulation or CD40-mediated activation renders human B cells to gain regulatory properties<sup>12,15,16</sup>. In addition, B cells that receive signals only through TLR9 could also regulate the differentiation and function of DCs<sup>46,47</sup>. All these results suggest that regulatory B cells could suppress the differentiation, maturation and function of DCs, and consequently could play a role in immune tolerance and in preventing inflammatory responses similar to those reported in the case of Tregs. However, our recent results also suggested that the repercussion of DC-B cell cross-talk on DC functions might depend on the type of activation signals received by B cells<sup>20</sup>, wherein we found that B cells that received

activation signals via BCR alone were not capable of blocking the differentiation of monocyte-derived DCs and did not inhibit TLR-mediated maturation of DCs. These findings are supported by previous reports that have revealed that based on the activation status, B cells might exert either tolerogenic or stimulatory effects on immune cells<sup>48,49</sup>.

Here we show that B cells pre-activated with either BCR or CD40 stimulation, or both, are capable of inducing maturation of DCs. Further, we found that contact-dependent interaction mediated by B-cell activation-associated molecules such as CD69, BAFF-R and TACI is involved in DC maturation. Interestingly, several studies have shown that activation-associated molecules of T cells and NKT cells, such as CD69 and CD40L, could modulate maturation and function of APCs, including DCs, monocytes and macrophages<sup>50,51</sup>. CD69 is shown to be involved in the regulation of sphingosine 1-phosphate receptor-1-mediated DC migration into lymph nodes and lymphocyte egress from lymph nodes<sup>52</sup>. Although till date the receptor binding to CD69 is elusive, it could be speculated that B cells on receiving activation signals, upregulate CD69, which



**Figure 8 | Th2 polarization by B-cell-matured DCs is dependent in part on OX-40 ligand.** Immature DCs were cultured for 48 h in the medium containing granulocyte macrophage colony-stimulating factor and IL-4 alone ( $DC_{ctrl}$ ), or co-cultured at 1:1 ratio with  $CD19^+$  B cells that were either in resting phase ( $DC_{Rest-B}$ ) or directly activated in DC-B cell co-culture via BCR stimuli ( $DC_{BCR-B}$ ). **(a,b)** Supernatants of DC-B cell co-cultures were analysed for IL-12p70 and IL-33. Mean  $\pm$  s.e.m. of data from five to ten donors. **(c,d)** Expression of CD70 and OX-40L on DCs that were gated negative for CD20. Representative plot and mean  $\pm$  s.e.m. of data from ten donors. **(e)** Experimental design for OX-40L blocking experiments and repercussion on T-cell cytokines. B-cell-matured DCs were purified from the DC-B cell co-cultures by depleting B cells.  $CD4^+CD45RO^-$  naive Th cells were co-cultured with these B-cell-matured DC at the ratio of 20:1 for 6 days ( $T_{BCR-B-DC}$ ) either alone or in the presence of isotype IgG antibody (+ Isotype IgG) or blocking antibody to OX-40L (+ Anti-OX-40L). **(f,g)**  $CD3^+$  T cells were analysed for intracellular expression of cytokines or Ki-67 by flow cytometry and **(h)** supernatants from DC-T cell co-cultures were analysed for different T-cell cytokines. Representative plot and mean  $\pm$  s.e.m. of data from five donors. \* $P < 0.05$ ; \*\*\* $P < 0.001$  NS, not significant by one-way analysis of variance test.

interacts with surface receptor on DCs to induce activation. However, more studies are needed to clarify the role of CD69 in B-cell-mediated regulation of DC maturation and function. Interaction of TACI on B cells with membrane-bound BLyS (B-lymphocyte stimulator)/BAFF on DCs was shown to be important for priming naive  $CD8^+$  T cells *in vivo*<sup>53</sup>, thus supporting our findings on the role of TACI and BAFF-R in DC activation. Notably, despite lower expression of these activation molecules, B cells stimulated with CD40 or CD40L + IL-4 induced DC maturation similar to BCR signalling (Supplementary Fig. 9). Interestingly, although activated naive ( $CD27^-CD19^+$ ) as well as memory ( $CD27^+CD19^+$ ) B cells were capable of inducing DC activation, our preliminary results indicate that naive B cells, but not memory B cells, require both BCR as well as CD40 signalling to be able to induce DC maturation. Based on these findings we believe that in addition to TACI, BAFF-R and CD69, other B-cell surface molecules, yet to be identified, are also required for the induction of DC maturation by activated B cells.

Activated T cells, innate lymphocytes and neutrophils have been shown to induce maturation of DCs with Th1 cell polarization. Further, Th1 polarization was found to be due to enhanced secretion of IL-12p70 by DCs, which is facilitated at least in part by  $IFN-\gamma$  secretion, in case of T and NK cells, and by  $TNF-\alpha$  production in case of neutrophils<sup>8–11</sup>. On the other hand, in our study, we found that B-cell-matured DCs did not display significant amount of Th1-inducing molecules such as IL-12p70 and CD70, which explains the unchanged Th1 differentiation. Similarly, lack of specific polarizing cytokines could be the possible reason for B-cell-matured DCs not to favour the differentiation of Th17 cells (IL-1 $\beta$ ), follicular Th (T<sub>fh</sub>) cells (IL-12 and IL-21) and Tregs (TGF- $\beta$ ). However, B-cell-matured DCs secreted moderate amount of IL-6 and  $TNF-\alpha$ , which are the polarizing cytokines for human Th22 (ref. 54). These cytokines could be responsible for the moderate increase in IL-22 expression in T cells by B-cell-matured DCs.

Development of Th2 cells is facilitated by various cytokines, such as IL-4, TSLP and IL-33, in the absence of  $IFN-\gamma$  and IL-12

signalling<sup>26,55,56</sup>. It is well known that DCs do not contribute to Th2 polarization by secreting IL-4. However, T-cell stimulation by DCs in the presence of IL-4 contributed by other immune cells such as basophils, mast cells, eosinophils and naive CD4<sup>+</sup> T cells could support Th2 differentiation<sup>55</sup>. DCs could also induce IL-4 production from some of these cells via IL-33. It was demonstrated that IL-33 acts on mast cells via ST2 and activates nuclear factor- $\kappa$ B and mitogen-activated protein kinase<sup>57</sup>. Alternatively, IL-33 could directly initiate the production of Th2 cytokines from polarized Th2 cells *in vitro* and promote IL-4, IL-5 and IL-13 *in vivo*<sup>57</sup>. However, we could not detect IL-33 in the culture supernatant of B-cell-matured DCs, thus ruling out a role for IL-33-mediated Th2 polarization by B-cell-matured DCs.

In addition to cytokines, signalling mediated by interaction of OX-40L, one of the members of the TNF receptor superfamily molecules on DCs, with OX-40 on T cells could also favour Th2 cell responses<sup>58–62</sup>. DCs do not express OX40L constitutively, rather expression of which is induced when cells sense activation signals from antigens. Similarly, naive CD4<sup>+</sup> T cells lack the expression of OX-40 and is transiently induced on these cells when they receive activation signals from APCs<sup>63</sup>. We found that along with other co-stimulatory molecules of B7 family, activated B cells significantly upregulated the expression of OX-40L on DCs, which was similar to TSLP–DC. The interaction of these B7 co-stimulatory molecules with CD28 on T cells could sustain the expression of OX-40 on T cells<sup>63</sup>. Of note, blocking OX-40L on DCs significantly inhibited the secretion of Th2 cytokines IL-4, IL-5 and IL-13. Our results thus demonstrate that B-cell-matured DCs could induce Th2 response at least in part through OX-40L. Interestingly, DC-produced IL-6 was also shown to promote Th2 differentiation by inducing STAT3-mediated c-Maf and NFATc2 expression<sup>64–67</sup>, and could interfere with development of Th1 cells by upregulating suppressor of cytokine signalling 1 expression<sup>68</sup>. In this context, in addition to OX-40L, IL-6 produced by B-cell-matured DCs might also be involved in the induction of Th2 cells.

We also show that Th2 cells differentiated by B-cell-matured DCs are capable of stimulating IgE production in B cells, similar to TSLP–DC-activated T cells. In fact, previous studies have shown that IL-4 and IL-13 secreted by Th2 cells mediate IgE responses from B cells<sup>69,70</sup>. Therefore, based on these reports, it is likely to be that IL-4 and/or IL-13, secreted by Th2 cells that were differentiated by B-cell-matured DCs, are involved in the production of IgE from B cells. However, further investigations are required to confirm these preliminary results.

In summary, our results indicate that B cells have the ability to control their own effector functions by enhancing Th2-differentiation ability of human DCs. However, promotion of Th2 polarization by B cells via DCs might act as double-edged sword. In case of infection, differentiated Th2 cells might support the production of antibodies from B cells and to neutralize the pathogens and their derived products. Conversely, in autoimmune and allergic diseases, Th2 cells that were polarized by B-cell-matured DCs could amplify autoimmune/inflammatory responses by providing help for autoantibody/IgE-producing B cells. Further, by enhancing the production of chemokines CCL17 and CCL22 in DCs, activated B cells might also promote recruitment of Th2 cells. CCL17 and CCL22 are known to recruit CCR4<sup>+</sup> Tregs as well and are expected to act as inflammation-limiting factor in the initial phase of immune response. However, it is well established that Tregs are incompetent to regulate sustained chronic inflammation. Under such inflammatory conditions as in the case of lupus, stimulation of B-cell-derived IL-10 production by CpG-DNA and activation of regulatory B cells could act as inflammation-limiting factor by suppressing the BCR/CD40-activated B-cell-mediated maturation of DCs.

## Methods

**Reagents and antibodies.** The medium used throughout was RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated (56 °C, 30 min) FCS, 100 U ml<sup>-1</sup> penicillin and 100 µg ml<sup>-1</sup> streptomycin. F(ab')<sub>2</sub> fragments of affinity-purified goat anti-human IgM (Fc fragment-specific) was obtained from Jackson ImmunoResearch Laboratories (West Grove, USA). Cell purification reagents such as microbeads and isolation kits were from Miltenyi Biotec (Paris, France).

**Purification of cells.** Peripheral blood mononuclear cells (PBMCs) were obtained from buffy bags of healthy donors by Ficoll density gradient centrifugation. Buffy bags of the healthy blood donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang, Paris, France. Ethical committee permission was obtained for the use of buffy bags of healthy donors (N°12/EFS/079). B cells were isolated from PBMCs by positive selection using the human CD19 microbeads and monocytes were purified by using human CD14 microbeads. Monocytes (0.5 × 10<sup>6</sup> cells per ml) were cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF; 1,000 IU per 10<sup>6</sup> cells) and IL-4 (500 IU per 10<sup>6</sup> cells) (both cytokines from Miltenyi Biotec) for 5–7 days to obtain immature monocyte-derived DCs.

CD4<sup>+</sup> T cells were either selected positively by CD4 microbeads or by negative selection using CD4<sup>+</sup> T-cell isolation kit II. Further, from the negatively selected T cells, naive CD4<sup>+</sup> T cells were isolated by selectively depleting CD45RO<sup>+</sup> T cells by using CD45RO microbeads. The purity of the separated cells was > 95% in all the populations.

**B-cell stimulation.** Freshly isolated B cells were used as 'resting B cells' for co-culture with DCs. For pre-activation, B cells (0.5 × 10<sup>6</sup> cells per ml) seeded in U-bottom 96-well plates were stimulated via BCR using F(ab')<sub>2</sub> of goat anti-human IgM (10 µg ml<sup>-1</sup>) or with CD40-mediated signalling using agonistic anti-human CD40 monoclonal antibody (mAb; mouse IgG2B clone 82111, R&D Systems, Lille, France; 5 µg ml<sup>-1</sup>) or with BCR + CD40 or recombinant CD40 ligand (CD40L, R&D Systems; 5 µg ml<sup>-1</sup>) + IL-4 (500 U ml<sup>-1</sup>) for 48 h to obtain 'activated B cells'. The agonistic nature of anti-CD40 antibodies was confirmed by various approaches. Anti-CD40 mAbs induced clustering, proliferation and activation of B cells. In a synergistic manner, anti-CD40 agonistic antibodies enhanced B-cell activation and proliferation induced by BCR signalling. Moreover, B cells stimulated with anti-CD40 agonistic antibodies induced significant proliferation of allogeneic T cells.

Supernatants from activated B-cell cultures were collected and centrifuged at 10,000 r.p.m. for 10 min to remove cellular debris. Before co-culture with immature DCs, both resting and pre-activated B cells were treated with mitomycin C (50 µg ml<sup>-1</sup>; Sigma-Aldrich, Saint-Quentin Fallavier, France) or paraformaldehyde fixed (1% in PBS for 30 min at 4 °C) and washed thoroughly to generate mitotically inactive cells to keep the B-cell number constant in B cell–DC co-culture systems.

**B cell–DC co-cultures.** Immature monocyte-derived DCs (0.1 × 10<sup>6</sup> cells per 200 µl per well) with GM-CSF and IL-4 were cultured either alone or with B cells (resting or pre-activated) at the ratio of 1:1 in U-bottom 96-well plates for 48 h. In some experiments, DCs were stimulated with recombinant human TSLP (20 ng per 0.1 × 10<sup>6</sup> cells, R&D Systems). For transwell experiments, B cells and DCs were kept separated by a 0.4-µm membrane. DCs (0.5 × 10<sup>6</sup> in 600 µl) were placed in the lower chamber of the transwell plate and pre-activated B cells (0.5 × 10<sup>6</sup> in 100 µl) in the upper chamber. For the stimulation of DCs using supernatant from activated B cells, immature DCs (0.1 × 10<sup>6</sup> cells per 250 µl total volume per well) were cultured in GM-CSF and IL-4 alone, or with supernatant from activated B cells (200 µl per well).

In some experiments, resting B cells were directly stimulated in DC–B cell co-culture by using F(ab')<sub>2</sub> of goat anti-human IgM (10 µg ml<sup>-1</sup>). In these experiments, the role of various surface molecules in contact-dependent induction of maturation of DCs by activated B cells was explored by employing blocking mAbs to anti-CD69 (10 µg ml<sup>-1</sup>), anti-TACI (10 µg ml<sup>-1</sup>), anti-CD54 (10 µg ml<sup>-1</sup>), anti-BAFF-R (10 µg ml<sup>-1</sup>) and anti-CD11a (10 µg ml<sup>-1</sup>), or mouse IgG isotype control antibodies (all from R&D systems).

**DC–T cell co-culture and mixed lymphocyte reaction.** Following 48 h of B cell–DC co-culture, B cells were depleted using CD20 microbeads and B-cell-depleted DCs were used for the co-culture with T cells. The purity of DCs following B-cell depletion was up to 98%.

For mixed lymphocyte reaction, graded doses of B-cell-depleted DCs were co-cultured in U-bottom 96-well plate with allogeneic total CD4<sup>+</sup> T cells (0.05 × 10<sup>6</sup> per well per 200 µl) in complete RPMI-1640 medium supplemented with 10% human AB serum. After 4 days of culture, 50 µl of supernatants were collected from each well before pulsing cells with 1 µCi (0.037 MBq) [<sup>3</sup>H]thymidine overnight to quantify T-cell proliferation. Radioactive incorporation was measured by standard liquid scintillation counting and results were expressed as counts per minute.

For analysing DC-mediated T-cell polarization, DCs were seeded with CD45RO<sup>-</sup> naive Th cells (0.1 × 10<sup>6</sup> per well per 200 µl) in the ratio of 1:20 in U-bottom 96-well plate. For blocking experiments, B-cell-depleted DCs were

pre-incubated with either isotype control antibody or anti-OX40L ( $10 \mu\text{g ml}^{-1}$ ) for 30 min before co-culture with T cells. Following 6 days, cell-free supernatants were collected to analyse various T-cell cytokines by enzyme-linked immunosorbent assay. Cells were split into two parts and one part of cells was stained for surface molecules and intracellular T-cell transcription factor. The other part was restimulated for intracellular cytokine staining.

**Cytokine and chemokine assay.** BD Cytokine Bead Array (CBA) kits (BD Biosciences, Paris, France) for human inflammatory cytokines (IL-1 $\beta$ , CXCL8/IL-8, TNF- $\alpha$ , IL-6, IL-10 and IL-12p70) and human Th1/Th2 cytokines (IL-2, IL-4, IL-5, IL-10, TNF- $\alpha$  and IFN- $\gamma$ ) were used to determine the cytokine levels in the supernatants. IL-33 and IL-17A, and chemokines CCL3, CCL4, CCL17, CCL18, CCL22, CXCL9, CXCL10 and CXCL16 were quantified using DuoSet ELISA kit (R&D Systems). IL-13, IL-17F and IL-22 were analysed using ELISA Ready-SET-Go kits (eBioscience, Paris, France). IL-21 was determined using ELISA MAX Deluxe Sets (BioLegend, San Diego, USA).

**Flow cytometry.** The following fluorochrome-conjugated antibodies were used at a dilution of 1 in 50. BD Biosciences: fluorescein isothiocyanate-conjugated antibodies to CD1a, CD86, HLA-DR, CD71, IFN- $\gamma$  and Foxp3; PE-conjugated antibodies to CD25, CD83, CD80, TACI and IL-4; APC-conjugated antibodies to CD19, CD20 and CD40L; Pacific blue-conjugated antibodies to CD4 and CD20; anti-CD69-APC-Cy7; anti-CCR4-PE-Cy7; and anti-CXCR3-AF700. Antibodies from eBioscience included CD40-APC, CD1a-Pacific Blue, CXCR5-PE, IL-6-APC, IL-17A-PE, TACI-PE, BAFF-R-fluorescein isothiocyanate, TNF- $\alpha$ -AF700, IFN- $\gamma$ -APC-eFluor 780, GATA3-PerCP-eFluor 710, T-bet-PE-Cy7, RORC-APC and Foxp3-APC. In addition, antibodies to CD3 (BV605), helios (22F6), CD11c (BV711), CD20 (BV650), CD27 (PE-Cy7), CD80 (BV605), CD206 (APC-Cy7), DC-SIGN (PE-Cy7), CCR7 (BV711), CCR6 (BV605), Ki-67 (BV711), IL-2 (BV650), IL-4 (PE), IL-17A (BV421), OX-40L (PE) and IL-8 (PerCP-Cy5.5) from BioLegend and anti-CD40-PE from Beckman Coulter (Villepinte, France) were also used.

Cells for surface staining were suspended in 10% FCS/PBS and antibodies against surface molecules were added at pre-determined concentration and incubated for 20 min at 4 °C. After washing, cells were fixed with BD Fix buffer and washed before analysis on flow cytometer. The data are presented as percentage of cells positive for indicated molecules or mean fluorescence intensities (MFI) of their expression.

For intracellular staining, DC-T cell co-cultures were stimulated with phorbol myristate acetate ( $50 \text{ ng ml}^{-1}$ ; Sigma-Aldrich) and ionomycin ( $500 \text{ ng ml}^{-1}$ ; Sigma-Aldrich) at 37 °C for 5 h in the presence of BD golgistop and BD golgiplug during the last 4 h. Following surface staining, cells were fixed and permeabilized (Foxp3 Fixation/Permeabilization kit, eBioscience) according to the manufacturer's instructions, and incubated at room temperature with fluorescence-conjugated mAbs. Cells were acquired on LSRII and processed with FACS DIVA software (BD Biosciences) and analysed by Flowjo.

**IgE production by B cells.** CD19<sup>+</sup> B cells were isolated from PBMC and were cultured in RPMI-1640–10% FCS either alone ( $0.02 \times 10^6$  cells per well per 200  $\mu\text{l}$ ) in U-bottomed 96-well plates or with T cells at 1:2.5 ratio for 12 days. Cell-free culture supernatants were collected and quantity of IgE was measured by enzyme-linked immunosorbent assay (Bethyl Laboratories, Euromedex, Soufflweyersheim, France).

**Statistical analysis.** One-way analysis of variance (repeated measures with Tukey's multiple comparison test) was used to determine the statistical significance of the data.  $P < 0.05$  was considered significant.

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## Acknowledgements

This study was supported by the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement HEALTH-2010.2.4.5-2 ALLFUN. We also thank the Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie and Université Paris Descartes for support.

## Author contributions

M.S.M. and J.B. conceived the study and designed the experiments. M.S.M., M.S., P.H. and E.S.-V. performed experiments. M.S.M., M.S., P.H., E.S.-V., S.V.K. and J.B. analysed the results. B.P. provided the advice. M.S.M. and J.B. wrote the paper.

## Additional information

**Supplementary Information** accompanies this paper at <http://www.nature.com/naturecommunications>

**Competing financial interests:** The authors declare no competing financial interests.

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**How to cite this article:** Maddur, M. S. *et al.* Human B cells induce dendritic cell maturation and favour Th2 polarization by inducing OX-40 ligand. *Nat. Commun.* **5**:4092 doi: 10.1038/ncomms5092 (2014).

## LETTERS

DOI 10.1002/art.38683

### Interferon- $\alpha$ inhibition by intravenous immunoglobulin is independent of modulation of the plasmacytoid dendritic cell population in the circulation: comment on the article by Wiedeman et al

To the Editor:

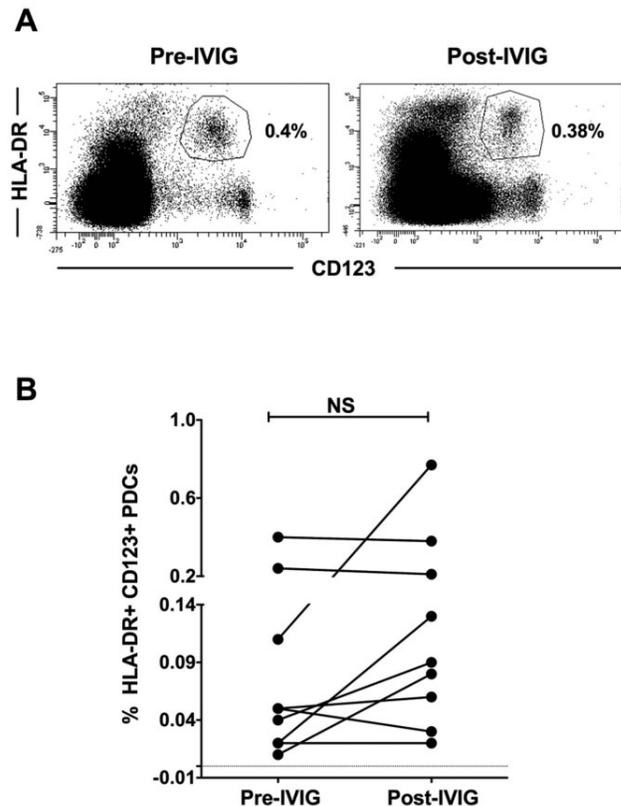
High-dose intravenous immunoglobulin (IVIG) is used in the therapy of various rheumatic diseases, and the beneficial effects of IVIG in these autoimmune and inflammatory conditions are mediated through several mutually nonexclusive mechanisms (1–3). Recent data reported by Wiedeman et al (4) suggest that one such action of IVIG comprises inhibition of interferon- $\alpha$  (IFN $\alpha$ ) production by two distinct mechanisms. The first mechanism described by Wiedeman and colleagues involved Fc $\gamma$ -mediated inhibition of immune complex binding to Fc $\gamma$  receptor IIa on plasmacytoid dendritic cells (PDCs). The second mechanism involved F(ab')<sub>2</sub> fragment-dependent inhibition of IFN $\alpha$  production when PDCs were stimulated with Toll-like receptor 7 (TLR-7) and TLR-9 agonists. Those authors also reported that the inhibitory effect of IVIG on IFN $\alpha$  production by TLR-stimulated PDCs required monocyte-derived prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (4). These data, along with findings described in a previous report on the inhibitory effect of IVIG on IFN $\alpha$ -mediated differentiation of monocyte-derived DCs (5), suggest that IVIG affects IFN $\alpha$ -mediated inflammatory pathways. The inhibitory effect of IVIG on IFN $\alpha$  production reported by Wiedeman et al also raises another possibility, that this inhibition might be due to a reduction in the number of PDCs, the principal producers of IFN $\alpha$ .

PDCs and type I IFN are implicated in the pathogenesis of various rheumatic diseases, including systemic lupus erythematosus, myositis, rheumatoid arthritis, and psoriasis (6,7). Aberrant activation of PDCs and their migration to inflamed tissue, and high levels of type I IFN, are hallmarks of these diseases. Ablation of PDCs in vivo was found to inhibit autoimmunity via expansion of myeloid-derived suppressor cells (8). In addition, antiinflammatory agents, such as corticosteroids in high doses (1 gm/day), are known to reduce the number of circulating PDCs (9). We therefore investigated whether the inhibitory effects of IVIG on IFN $\alpha$  production reported by Wiedeman et al also implicate modulation of the circulating PDC population in vivo in patients with rheumatic disease.

Heparinized blood samples were obtained from 9 patients with myositis (7 female and 2 male; ages 27–70 years), before and 48–72 hours after initiation of IVIG (1 gm/kg). All patients provided written informed consent for participation in the study, and ethics committee permission was received prior to study initiation. The specific diagnoses of the patients were as follows: polymyositis (n = 3), dermatomyositis (n = 1), anti-signal recognition particle-associated necrotizing myopathy (n = 2), anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase-associated necrotizing myopathy (n = 2), and anti-Mi2-associated unclassified myositis (n = 1). Additional treatments patients were receiving included methotrexate and

prednisone. PDCs in whole blood were analyzed by flow cytometry using surface expression of HLA-DR and CD123 (Figure 1A).

Before IVIG therapy, the mean  $\pm$  SD percentage of circulating PDCs among total blood leukocytes in the myositis patients was  $0.104 \pm 0.132\%$ . After IVIG therapy, we observed a marginal increase in PDCs in 4 of the patients, probably indicating the inhibitory effects of IVIG on the migration of PDCs toward inflamed tissue. However, overall, IVIG therapy did not lead to significant alterations in circulating PDC



**Figure 1.** Effect of intravenous immunoglobulin (IVIG) on circulating plasmacytoid dendritic cells (PDCs) from patients with myositis. Heparinized blood samples were obtained 48–72 hours after initiation of high-dose IVIG therapy. Red blood cells were separated from nucleated cells using HetaSep (StemCell Technologies) (1 part HetaSep, 5 parts blood). **A**, Representative dot plots showing the percentage of PDCs with positive gating for HLA-DR and CD123. **B**, Changes in the percentage of HLA-DR+CD123+ PDCs in the circulation of myositis patients (n = 9) following IVIG therapy. Each symbol represents an individual patient. PDCs were analyzed by flow cytometry (LSR II; BD Biosciences) using fluorescence-conjugated monoclonal antibodies to HLA-DR (BD Biosciences) and CD123 (eBioscience). Statistical significance was assessed by Student's paired 2-tailed *t*-test. NS = not significant.

numbers, which were a mean  $\pm$  SD of  $0.197 \pm 0.242\%$  of total leukocyte numbers after treatment ( $P = 0.249$ ) (Figure 1B). These data, along with those reported by Wiedeman et al (4), thus suggest that although IVIG inhibits IFN $\alpha$  production from PDCs via monocyte-derived PGE<sub>2</sub>, this reduction in IFN $\alpha$  production is not due to an alteration in the number of circulating PDCs in vivo. Importantly, it has been shown that IVIG could also induce cyclooxygenase 2–dependent PGE<sub>2</sub> from human DCs (10), which would lead to an expansion of CD4+CD25+FoxP3+ Treg cells.

Supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie, and Université Paris Descartes.

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DOI 10.1002/art.38684

## Reply

To the Editor:

Sharma et al present the results of their studies on the effect of IVIG on circulating PDCs in patients with myositis. Based on our reported finding that IVIG inhibits PDC production of IFN $\alpha$  in vitro, they offer two possible hypotheses on how IVIG may affect PDCs in vivo. One possibility is that IVIG treatment would simply reduce PDC numbers. Alternatively, IVIG inhibition of PDCs may reduce their activation and subsequent migration to inflamed tissue, thus resulting in increased numbers of PDCs in the periphery. By comparing the percentage of PDCs in peripheral blood before, and then 2–3 days after, high-dose IVIG therapy, they found that the peripheral PDCs were slightly, but not statistically significantly, increased. These results indicate that IVIG does not induce cell death of PDCs.

We find these results of interest as they demonstrate what we would expect to see in vivo based on our observation that IVIG alters the functional properties of PDCs. We also considered death of PDCs as a potential mechanism by which IVIG could inhibit IFN $\alpha$  production. However, we found that IVIG treatment of lupus immune complex-stimulated PDCs did not increase cell death after 22 hours of culture (Figures 1A and B). As reported in our article, we had shown that in response to TLR ligand stimulation of IFN $\alpha$ , the sialylated subset of IVIG (sialic acid-specific *Sambucus nigra* agglutinin positive) was a more potent inhibitor. Even so, treatment with this IVIG subset did not result in increased PDC death in vitro (Figure 1C). These results are consistent with the maintenance of PDC numbers after IVIG treatment in vivo observed by Sharma et al.

While the number of peripheral PDCs is unaltered with IVIG treatment, it would be of great interest to determine whether high-dose IVIG regulates IFN $\alpha$  production in vivo. Increased serum IFN $\alpha$  has been linked to both myositis and systemic lupus erythematosus, and implicated in their pathogenesis (1,2). It would be relatively straightforward to test

## Correspondence

**GM-CSF along with IL-4 but not alone is indispensable for the differentiation of human dendritic cells from monocytes**

To the Editor:

López-Bravo et al<sup>1</sup> reported that IL-4 blocks T<sub>H</sub>1-polarizing/inflammatory cytokine expression during murine monocyte-derived dendritic cell (Mo-DC) differentiation. For their experimental setup, authors cultured monocytes in the presence of GM-CSF alone for 24 hours to obtain control Mo-DCs or with IL-4 to derive IL-4-Mo-DCs. They concluded that IL-4 during murine Mo-DC differentiation prevent the ability of LPS-stimulated Mo-DCs to produce T<sub>H</sub>1-polarizing and inflammatory cytokines. To obtain translational insight on this report, we investigated whether the Mo-DC differentiation condition and duration, and conclusion made by López-Bravo et al, is also pertinent in human Mo-DCs.

We differentiated CD14<sup>+</sup> human monocytes with GM-CSF alone or with IL-4 for 24 hours (see the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) as described by López-Bravo et al. In contrast to mice, human monocytes did not differentiate into DCs in 24 hours in either of the conditions (Fig 1, A and B). Most of the cells remained positive for CD14, and only 2% to 3% of the cells were CD1a<sup>+</sup>, a differentiation marker for human DCs. We then followed the kinetics of Mo-DC differentiation over 5 days.<sup>2</sup> Monocytes differentiated with GM-CSF and IL-4 progressively lost CD14 and CD16 during this period. At day 5, differentiated DCs were either negative or very low positive for the above markers and were highly positive for CD1a (Fig 1, C). In contrast, cells differentiated in GM-CSF alone never acquired the DC phenotype even after 5 days: about 20% of the cells retained CD14 and were highly positive for CD16. The expression of CD1a was less than 10% (Fig 1, C). Thus, GM-CSF-differentiated cells exhibited phenotype similar to those of M1-type macrophages rather than DCs.<sup>3</sup> Of note, the expression of CD14 and CD16 was retained on these GM-CSF-differentiated M1-type cells even following LPS stimulation while they were negative on GM-CSF and IL-4-differentiated DCs (Fig 1, D and E).

As in the study by López-Bravo et al, GM-CSF-differentiated cells produced large quantities of inflammatory cytokines on LPS stimulation (Fig 1, F). But unlike mice, these data only indicate differences between human M1 macrophages and DCs but not 2 DC subsets in their ability to produce cytokines. The high proportion of inflammatory cytokines produced by GM-CSF-differentiated cells could also be attributed to CD14 molecules on these cells because CD14 acts as a coreceptor for LPS.<sup>4</sup> However, no major differences were observed in IL-10 and IL-12 secretion by these 2 cell types.

Pathogen-associated molecular patterns (PAMPs) have differential abilities to polarize T cells on interaction with DCs.<sup>5</sup> By using PAMPs of *Mycobacterium tuberculosis*, that is, mannosylated lipoarabinomannan (ManLAM), a glycolipid,<sup>6</sup> and Rv3812, a cell wall-associated protein belonging to proline-glutamic acid (PE)-polymorphic GC-rich repetitive-sequences (PGRS) family,<sup>7</sup> we examined the T-cell-polarizing capacity of GM-CSF-IL-4-differentiated Mo-DCs. While DCs stimulated with ManLAM polarized T<sub>H</sub>2 responses, this polarization could

be skewed toward T<sub>H</sub>1 when DCs were stimulated with a combination of ManLAM and Rv3812 (Fig 1, G). Thus, the presence of IL-4 during human Mo-DC differentiation does not block the ability of DCs to polarize T<sub>H</sub>1 responses; rather, it is dictated by PAMP-pattern recognition receptor interaction.

To conclude, although murine DCs could be obtained by culturing monocytes with GM-CSF alone for 24 hours, this condition is not applicable for humans because it will yield M1-type macrophages. Thus, GM-CSF and IL-4 are both indispensable for the differentiation of human Mo-DCs and these cells retain the ability to polarize T<sub>H</sub>1 responses depending on the stimuli they receive.

We thank the National Institutes of Health Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases, National Institutes of Health, for kindly providing ManLAM of *Mycobacterium tuberculosis*, strain H37Rv, NR-14848.

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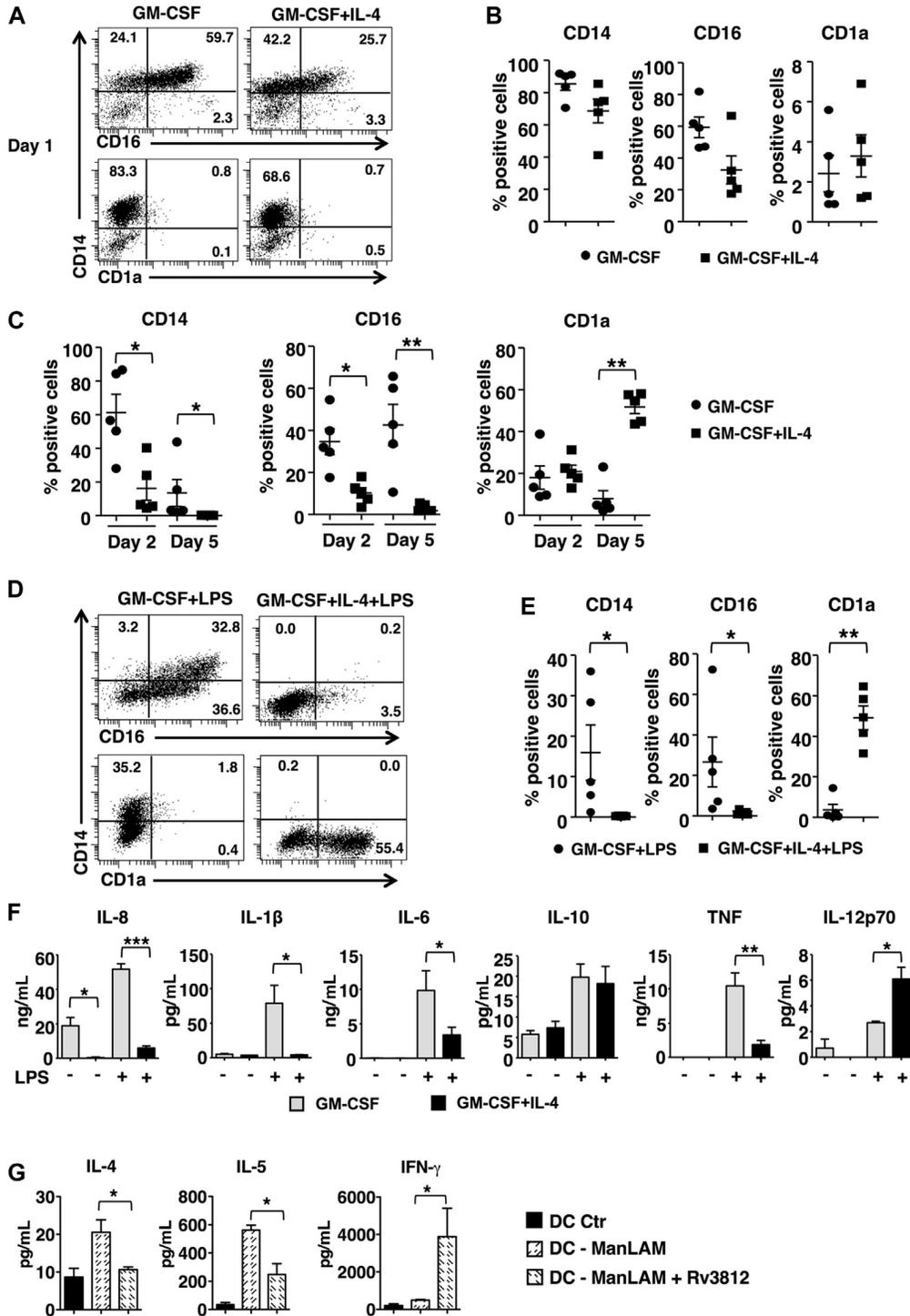
This study was supported by Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, Université Paris Descartes (S.V.K. and J.B.), Centre National de la Recherche Scientifique (S.V.K.), Indian Institute of Science, Department of Biotechnology, Department of Science and Technology, Council for Scientific and Industrial Research (K.N.B.), a grant from the Indo-French Center for Promotion of Advanced Research (CEFIPRA, Reference No. 4803-1) (J.B. and K.N.B.), fellowships from the Indian Institute of Science and CEFIPRA (S.H.), and a fellowship from CEFIPRA (M.S.).

Disclosure of potential conflict of interest: K. N. Balaji and J. Bayry have received research support from the Indo-French Center for Promotion of Advanced Research. The rest of the authors declare that they have no relevant conflicts of interest.

**Editor's note:** There is no accompanying reply to this correspondence.

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**FIG 1.** Both GM-CSF and IL-4 are indispensable for the human Mo-DC differentiation. **A** and **B**, Flow cytometric analysis of cells following 24 hours differentiation of monocytes in GM-CSF or GM-CSF + IL-4. **C**, Kinetics of expression of markers over 5 days of differentiation (n = 5). Phenotype (**D** and **E**) and cytokines (**F**) secreted by LPS-stimulated GM-CSF or GM-CSF+IL-4-differentiated cells (n = 5). **G**, T-cell-polarizing ability of GM-CSF+IL-4-differentiated DCs stimulated with ManLAM or ManLAM + Rv3812 (n = 3). \**P* < .05, \*\**P* < .01, and \*\*\**P* < .001.

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<http://dx.doi.org/10.1016/j.jaci.2014.02.021>

## METHODS

### Human monocyte differentiation

Human PBMCs were isolated from buffy coats of healthy blood donors purchased from Centre Necker-Cabanel (Etablissement Français du Sang, Paris, France). Ethical committee permission was obtained for the use of buffy coats of healthy donors (No. 12/EFS/079). Circulating monocytes were isolated from these PBMCs using CD14 microbeads (Miltenyi Biotec, Paris, France). The purity was more than 98%. Monocytes were cultured in RPMI-1640 medium containing 10% FCS for indicated days in the presence of recombinant human GM-CSF (1000 IU/10<sup>6</sup> cells) alone or with recombinant human IL-4 (500 IU/10<sup>6</sup> cells)<sup>E1</sup> (both from Miltenyi Biotec) and used for subsequent experiments and analysis.

### Stimulation of cells with LPS

Cells were harvested following differentiation of monocytes for 5 days either in GM-CSF alone or in GM-CSF and IL-4. The cells were washed and further cultured in respective cytokines and stimulated with LPS (100 ng/mL/0.5 × 10<sup>6</sup> cells, from *Escherichia coli*, Sigma-Aldrich, Lyon, France) for 24 hours. Cell culture supernatants were collected for analyzing cytokines, and the phenotype of cells were analyzed by flow cytometry.

### DC:CD4<sup>+</sup> T-cell cocultures

DCs were derived from differentiation of monocytes for 5 days in GM-CSF and IL-4. The cells were washed and were cultured (0.5 × 10<sup>6</sup> cells/mL) with GM-CSF and IL-4 alone or GM-CSF, IL-4, 5 μg/mL of ManLAM (BEI resource, purified from H37Rv, NR-14848) or GM-CSF, IL-4, 5 μg/mL of ManLAM, 5 μg/mL of Rv3812 for 48 hours. Recombinant Rv3812 protein was obtained as previously described.<sup>E2</sup> Following extensive washings, DCs were cocultured with CD4<sup>+</sup> T cells at 1:20 for 96 hours. T-cell cytokines in DC:T cell cocultures were analyzed in cell-free culture supernatants.

### Flow cytometry

Surface staining of cells was carried out by using fluorochrome-conjugated mAbs to CD14, CD16, and CD1a (all from BD Biosciences, Le Pont de Claix, France). Cells were further processed for flow cytometry wherein 5000 gated events were recorded for each sample acquired and the data were analyzed using FACSDiva software (BD Biosciences).

### Cytokine analysis

Cytokines were quantified in cell-free culture supernatants using BD Cytometric Bead Array human inflammatory cytokine kits and human T<sub>H</sub>1/T<sub>H</sub>2 cytokine kits (BD Biosciences). The data were analyzed using FCAP Array software (BD Biosciences).

### Statistical analysis

Levels of significance for comparison between samples were determined by 2-tailed nonparametric Mann-Whitney test (for data depicted in figures as the median along with an appropriate range values) or by Student *t* test (for data depicted as means and SDs).<sup>E3</sup> Values of *P* < .05 were considered statistically correlated (\**P* < .05, \*\**P* < .01, \*\*\**P* < .001). All statistical analyses were performed using Prism 5 software (GraphPad Software, Inc, La Jolla, Calif).

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**blood**

2013 122: 1419-1427  
doi:10.1182/blood-2012-11-468264 originally published  
online July 11, 2013

## **Intravenous immunoglobulin expands regulatory T cells via induction of cyclooxygenase-2–dependent prostaglandin E2 in human dendritic cells**

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## IMMUNOBIOLOGY

Intravenous immunoglobulin expands regulatory T cells via induction of cyclooxygenase-2–dependent prostaglandin E<sub>2</sub> in human dendritic cellsJamma Trinath,<sup>1,2</sup> Pushpa Hegde,<sup>1,3</sup> Meenu Sharma,<sup>1,3</sup> Mohan S. Maddur,<sup>1,4,5</sup> Magalie Rabin,<sup>1,4,6</sup> Jean-Michel Vallat,<sup>6</sup> Laurent Magy,<sup>6</sup> Kithiganahalli N. Balaji,<sup>2</sup> Srinivasa V. Kaveri,<sup>1,4,5,7</sup> and Jagadeesh Bayry<sup>1,4,5,7</sup>

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## Key Points

- IVIg expands Tregs in vitro and in vivo via induction of COX-2–dependent PGE<sub>2</sub> in DCs.
- These functions of IVIg are mediated in part via interaction of IVIg and F(ab')<sub>2</sub> fragments of IVIg with DC-SIGN on DCs.

**CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) play a critical role in the maintenance of immune tolerance. Intravenous immunoglobulin (IVIg), a therapeutic preparation of normal pooled human IgG, expands Tregs in various experimental models and in patients. However, the cellular and molecular mechanisms by which IVIg expands Tregs are relatively unknown. As Treg expansion in the periphery requires signaling by antigen-presenting cells such as dendritic cells (DCs) and IVIg has been demonstrated to modulate DC functions, we hypothesized that IVIg induces distinct signaling events in DCs that subsequently mediate Treg expansion. We demonstrate that IVIg expands Tregs via induction of cyclooxygenase (COX)-2–dependent prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in human DCs. However, costimulatory molecules of DCs such as programmed death ligands, OX40 ligand, and inducible T-cell costimulator ligands were not implicated. Inhibition of PGE<sub>2</sub> synthesis by COX-2 inhibitors prevented IVIg-mediated Treg expansion in vitro and significantly diminished IVIg-mediated Treg expansion in vivo and protection**

**from disease in experimental autoimmune encephalomyelitis model. IVIg-mediated COX-2 expression, PGE<sub>2</sub> production, and Treg expansion were mediated in part via interaction of IVIg and F(ab')<sub>2</sub> fragments of IVIg with DC-specific intercellular adhesion molecule-3-grabbing nonintegrin. Our results thus uncover novel cellular and molecular mechanism by which IVIg expands Tregs. (*Blood*. 2013;122(8):1419-1427)**

## Introduction

Intravenous immunoglobulin G (IVIg) is a therapeutic preparation of normal human Ig purified from the pooled plasma of several thousand healthy donors. IVIg is used for the replacement therapy of primary immunodeficiencies and in the treatment of various pathological conditions, including immune thrombocytopenia (ITP), Kawasaki disease, anti-neutrophil cytoplasmic antibody-associated vasculitis, and others.<sup>1-5</sup> Despite its therapeutic use for more than 3 decades, the mechanism by which IVIg benefits autoimmune patients with diverse pathogenesis is not completely understood. However, available data from patients and various experimental models indicate that IVIg can benefit patients with autoimmune and inflammatory diseases via several mutually nonexclusive mechanisms.<sup>1,5-13</sup>

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (Tregs) play a critical role in the maintenance of immune tolerance and prevention of autoimmunity.<sup>14</sup> Deficiency of Tregs or their defective functioning lead to autoimmune and inflammatory conditions, whereas expansion of Tregs and/or amelioration in their functions are associated with recovery from autoimmune diseases.<sup>14</sup> Of note, several recent reports

have demonstrated that IVIg expands Tregs.<sup>15-26</sup> However, the cellular and molecular mechanisms by which IVIg expands Tregs are relatively unknown. As Treg expansion in the periphery requires signaling by antigen-presenting cells such as dendritic cells (DCs)<sup>27,28</sup> and IVIg has been demonstrated to modulate DC functions,<sup>29-32</sup> we hypothesized that IVIg induces distinct signaling events in DCs. These IVIg-educated DCs subsequently mediate the expansion of Tregs.

In the present report, we demonstrate that IVIg expands Tregs via induction of cyclooxygenase (COX)-2–dependent prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) in human DCs. Inhibition of PGE<sub>2</sub> synthesis prevented DC-mediated Treg expansion by IVIg in vitro and significantly diminished IVIg-mediated Treg expansion in vivo in an experimental autoimmune encephalomyelitis (EAE) model. Modulation of IVIg-mediated Treg expansion by COX-2 inhibitor in vivo also had repercussions on the evolution of EAE. Further, we found that IVIg-mediated COX-2 expression and PGE<sub>2</sub> production were mediated in part via interaction of IVIg with DC-specific intercellular adhesion

Submitted November 19, 2012; accepted July 7, 2013. Prepublished online as *Blood* First Edition paper, July 11, 2013; DOI 10.1182/blood-2012-11-468264.

J.T., P.H., and M.S. contributed equally to this study.

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molecule-3-grabbing nonintegrin (DC-SIGN) on DCs. Our results thus uncover novel cellular and molecular mechanism by which IVIg expands human Tregs.

## Methods

### Cell culture reagents and antibodies

Fluorochrome-conjugated monoclonal antibodies (mAbs) to human CD4, CD25, DC-SIGN, HLA-DR, programmed death-1 (PD-1)-ligand 1 (PD-L1), PD-ligand 2 (PD-L2), OX-40L, and inducible T-cell costimulator (ICOS) ligand (ICOSL) and to mouse CD4 and CD25 were from BD Biosciences (Le Pont de Claix, France). Fluorochrome-conjugated mAb to Foxp3 and intracellular staining kit were from eBioscience (Paris, France). MAb to COX-2 and  $\beta$ -actin were from Cell Signaling Technology (Ozyme, Saibt Quentin Yvelines, France). CD14 magnetic beads, CD4<sup>+</sup> T cell isolation kit II, GM-CSF, and interleukin (IL)-4 were from Miltenyi Biotec (Paris, France). Blocking mAb to DC-SIGN and isotype control mAb were from R&D Systems (Lille, France).

### Generation of human DCs

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy bags of healthy donors purchased from Hôpital Hôtel Dieu, Etablissement Français du Sang, Paris, France. Institutional Review Board (INSERM-EFS) approval was obtained for use of buffy bags of healthy donors. Informed consent was obtained in accordance with the Declaration of Helsinki. CD14<sup>+</sup> monocytes were isolated from PBMC by using CD14 magnetic beads. The purity was >98%. Monocytes were cultured in RPMI-1640 medium containing 10% fetal calf serum for 6 days in the presence of cytokines GM-CSF (1000 IU/10<sup>6</sup> cells) and IL-4 (500 IU/10<sup>6</sup> cells) to obtain DCs and were used for subsequent experiments. DC purity was  $\geq$ 98%.

### Treatment of DCs and monocytes with IVIg

DCs ( $0.5 \times 10^6$ /mL) were cultured with cytokines GM-CSF (1000 IU/10<sup>6</sup> cells) and IL-4 (500 IU/10<sup>6</sup> cells) alone or with IVIg (Privigen, CSL Behring AG, Bern, Switzerland) (10 or 15 mg/mL) for 24 hours. Circulating blood monocytes ( $0.5 \times 10^6$ /mL) were cultured alone or with IVIg (15 mg/mL). The concentrations of IVIg used in these experiments were within the range of IVIg dose used in the patients and other experiments.<sup>19</sup> IVIg was dialyzed before use and tested negative for endotoxins. F(ab')<sub>2</sub> fragments of IVIg were prepared by pepsin digestion (2% weight to weight ratio; Sigma-Aldrich, Saint-Quentin Fallavier, France) followed by dialysis and protein G chromatography to isolate F(ab')<sub>2</sub> fragments. Fc-fragments of IVIg were obtained from Dr M. C. Bonnet (Institut Mérieux, Lyon, France). F(ab')<sub>2</sub> fragments and Fc-fragments of IVIg were used at equimolar concentrations. The equimolar concentration of human serum albumin (HSA; Laboratoire Française de Biotechnologies, Les Ulis, France) was used as an irrelevant protein control.

### Treatment of DCs with pharmacological inhibitor

Pharmacological inhibitors of COX-2 activity, NS-398 or celecoxib (both from Sigma-Aldrich), were reconstituted in dimethylsulfoxide (DMSO). DCs were treated with NS-398 at a concentration of 10  $\mu$ M and celecoxib at 25  $\mu$ M for 60 minutes followed by treatment with IVIg. The concentrations of NS-398 and celecoxib were chosen after careful titration experiments assessing the viability of the DCs.

### Blocking antibody experiments

DCs ( $0.5 \times 10^6$ ) were preincubated with blocking mAb to DC-SIGN (5  $\mu$ g) or isotype control mAb (5  $\mu$ g) for 60 minutes followed by treatment with IVIg (15 mg/mL) for 24 hours.

### Co-culture of DCs with CD4<sup>+</sup> T cells

CD4<sup>+</sup> T cells from PBMCs of healthy donors were isolated using CD4<sup>+</sup> T cell isolation kit II. DCs following appropriate treatment were extensively washed and were cocultured with  $0.2 \times 10^6$  CD4<sup>+</sup> T at DC/T-cell ratios of

1:10 in 96-well U-bottom plates. The DC and CD4<sup>+</sup> T cells were from unrelated donors and hence TCR and CD28 stimulations were not provided during coculture. However, effectively similar results were also obtained when autologous DC and CD4<sup>+</sup> T cells were cocultured in the presence of anti-CD3 and anti-CD28 co-stimulation. After 4 days, Tregs were analyzed by flow cytometry (LSR II; BD Biosciences) by using a combination of CD4, CD25, and Foxp3 molecules.

### Flow cytometry

Surface staining of DC and T cells was carried out by using fluorochrome-conjugated mAbs. For intracellular staining of Tregs, cells were fixed, permeabilized, and incubated at room temperature with fluorochrome-conjugated mAb to Foxp3. Five thousand cells were acquired for each sample and the data were processed by using FACS DIVA software (BD Biosciences).

### Immunoblotting analysis of COX-2

DC pellets were lysed in RIPA buffer constituting 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/mL each of aprotinin, leupeptin, and pepstatin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 mM NaF. Following protein concentration estimation, equal amounts of proteins from each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Migrated proteins were then transferred onto polyvinylidene difluoride membranes by semidry western blotting method. Membranes were treated with 5% nonfat dry milk powder in Tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.1% Tween 20) for 60 minutes to prevent nonspecific binding of detecting antibodies. The blots were incubated with COX-2 antibody in TBST with 5% bovine serum albumin (BSA). After washing with TBST, blots were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody in TBST with 5% BSA for 2 hours. After further washing in TBST, the blots were developed with the ECL system (PerkinElmer) as per the manufacturer's instructions.  $\beta$ -Actin served as a loading control.

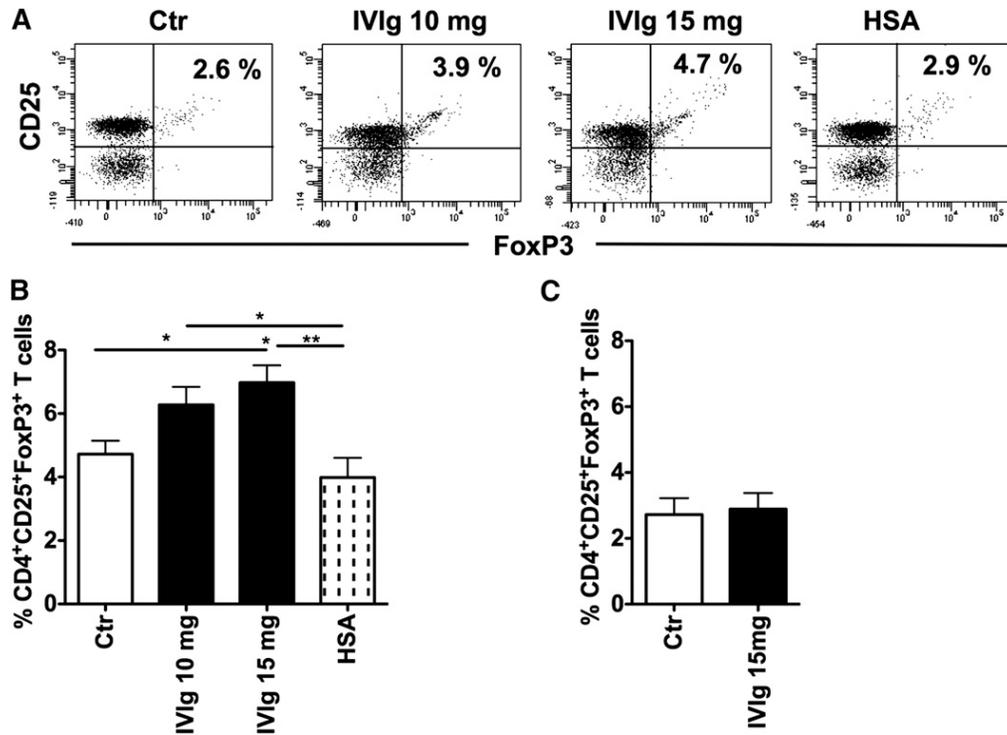
### EAE model and treatment with IVIg and NS-398

Animal studies were performed according to the guidelines of the Charles Darwin ethical committee for animal experimentation (Université Pierre et Marie Curie, Paris, France) at the pathogen-free animal facility of Centre de Recherche des Cordeliers, Paris. EAE was induced in 10-week-old female C57BL/6J mice (Janvier, Saint Berthevin, France) and evolution of disease was followed as previously reported.<sup>15,26</sup> EAE was induced in 3 different groups and each group included 6 to 8 mice. Mice were immunized with 200  $\mu$ g of MOG<sub>35-55</sub> peptide (MEVGGWYRSPFSRVVHLYRNGK; PolyPeptide laboratory, Strasbourg, France) emulsified in complete Freund's adjuvant (Sigma-Aldrich) containing 880  $\mu$ g of nonviable *Mycobacterium tuberculosis* H37RA. A final volume of 200  $\mu$ L was injected subcutaneously at 2 sites over the flanks. Additionally, 300 ng of pertussis toxin (List Biologic Laboratories, Meudon, France) was injected intravenously on the day of immunization and 48 hours later. IVIg was injected daily at 0.8 g/kg intraperitoneally from the day of the immunization until peak of the disease (day 16). NS-398 was intraperitoneally injected on every second day starting from the day of immunization until day 16 at 100  $\mu$ g/mouse. An equivalent volume and concentration of DMSO was injected to control mice and mice treated with IVIg.

### Isolation of mononuclear cells from the spleen and analysis of Tregs by flow cytometry

Mice were sacrificed during the onset of the EAE (day 12) by cervical dislocation and spleens were collected. Single cell suspensions were obtained by mechanical disaggregation and passing the cells through a 70- $\mu$ m nylon membrane filter. Red blood cells were lysed using ACK lysis buffer.

Mononuclear cells from spleen were treated with anti-mouse CD16/32 antibody (BD Biosciences) to block Fc-receptors and then surface labeled with anti-mouse CD4 and CD25 mAbs. Cells were then washed, fixed, permeabilized, and incubated at room temperature with fluorochrome-conjugated mAb to



**Figure 1. IVIg-treated human DCs but not monocytes expand Tregs.** (A) DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with IVIg (10 mg or 15 mg) or HSA for 24 hours. DCs were washed extensively and cocultured with CD4<sup>+</sup> T cells for 4 days. Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) were analyzed by flow cytometry. Representative dot plot of 8 independent experiments is shown. (B) Percentage (mean  $\pm$  SEM, n = 8) of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in the DC-CD4<sup>+</sup> T cell cocultures, \**P* < .05; \*\**P* < .01. (C) Circulating monocytes were cultured alone or with IVIg (15 mg) for 24 hours. Cells were washed extensively and cocultured with CD4<sup>+</sup> T cells for 4 days. Tregs (mean  $\pm$  SEM, n = 8) were analyzed by flow cytometry.

Foxp3. Ten thousand events were acquired for each sample and analyzed using BD LSR II and FACS DIVA software.

### Measurement of PGE<sub>2</sub>

PGE<sub>2</sub> in cell-free culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA). ELISA plates were incubated with test samples overnight at 4°C, followed by 3 washes with phosphate-buffered saline (PBS)-Tween (PBST). After blocking with 1% BSA in PBST for 1 hour at 37°C, wells were incubated with anti-PGE<sub>2</sub> antibodies (Sigma-Aldrich) for 6 hours at 37°C, followed by washing with PBST. The plates were incubated further with horseradish peroxidase-labeled anti-rabbit secondary antibody for 2 hours at 37°C, followed by development with 3,3',5,5'-tetra methylbenzidine (Sigma-Aldrich). The absorbance values were measured at 492 nm by using an ELISA reader.

### Statistical analysis

The significance of differences between series of results with 3 or more groups was assessed using the 1-way ANOVA and comparison between sets of results was assessed using Tukey post-test. Data with 2 groups were analyzed by 2-way Student *t* test. Values of *P* < .05 were considered statistically correlated (\**P* < .05; \*\**P* < .01; \*\*\**P* < .001). All statistical analyses were performed using Prism 5 software (GraphPad software).

## Results

### IVIg-treated human DCs expand Tregs

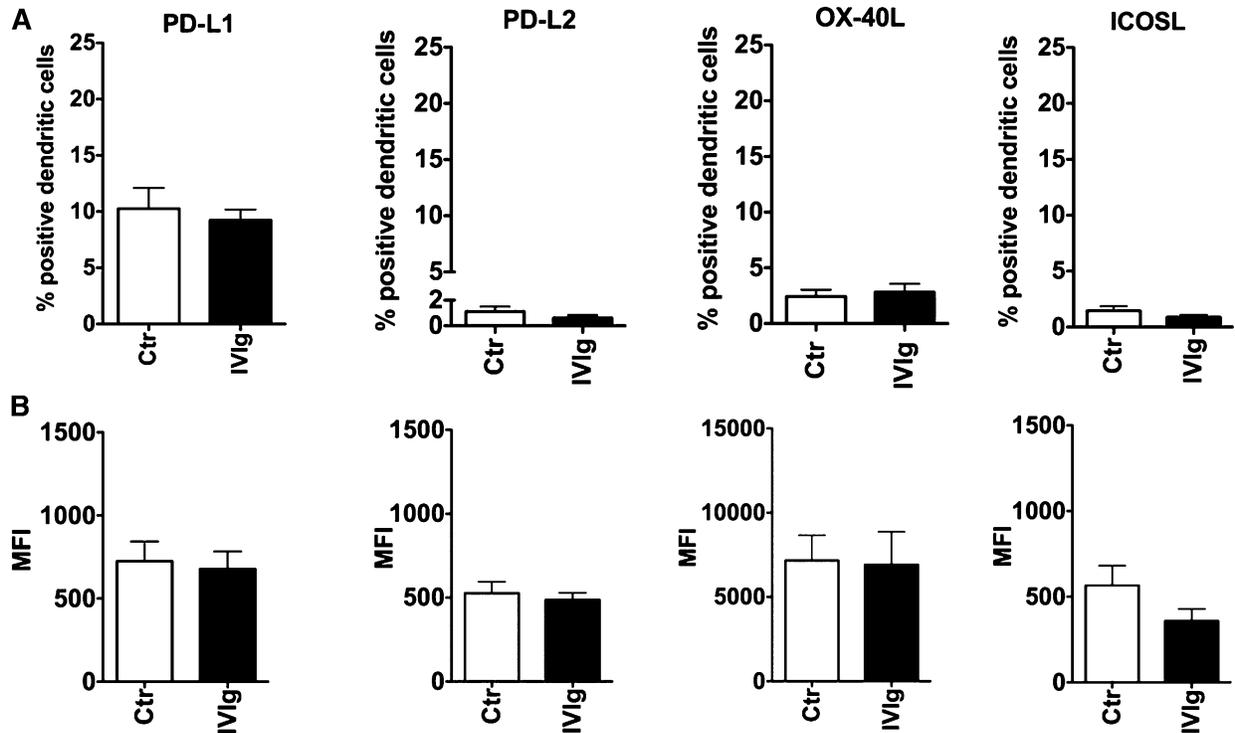
DCs have a unique ability to expand Tregs.<sup>27,28</sup> We therefore first aimed at confirming whether IVIg-treated human DCs expand Tregs. Six-day-old monocyte-derived DCs were treated with IVIg at 10- and 15-mg concentrations for 48 hours. The DCs were washed

extensively and then cocultured with CD4<sup>+</sup> T cells for 4 days and Treg expansion was analyzed. As compared with untreated DCs, IVIg-treated DCs significantly expanded CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>Tregs (Figure 1A-B). An equimolar concentration of HSA, used as an irrelevant protein control for IVIg, did not modify the ability of DCs to expand Tregs. Further, the effect of IVIg on Treg expansion was dose dependent. Nearly 20% more Tregs were expanded by 15 mg IVIg-treated DCs compared with 10 mg IVIg-treated DCs (Figure 1B). In contrast to DCs, IVIg-treated monocytes did not expand Tregs (Figure 1C), indicating that the effect of IVIg was specific for DCs. Together, these results imply that the Treg expansion observed in autoimmune patients following IVIg therapy is suggestive of the modulatory effect of IVIg on DCs.

### IVIg does not alter the expression of costimulatory molecules of DCs implicated in Treg expansion

We then aimed at identifying the mechanisms by which IVIg-treated DCs expand Tregs. The interaction of several CD4<sup>+</sup> T cell-DC costimulatory molecules, including PD-1 (CD279) receptor and its ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273); OX-40 and OX-40 ligand (OX-40L, CD252); and ICOS and ICOSL (CD275) could lead to Treg expansion.<sup>33-36</sup> Therefore, to explore whether IVIg-mediated Treg expansion implicates these costimulatory molecules on DCs, we analyzed the effect of IVIg on the expression of the above molecules on DCs.

We found that IVIg at a 15-mg concentration did not alter the basal expression of PD-L1 and PD-L2 on DCs, the ligands for PD-1 (*P* > .05) (Figure 2A-B). The expression level of both the molecules was on par with untreated DCs. Similarly, IVIg treatment did not modify the expression of OX-40L and ICOSL on DCs (Figure 2).



**Figure 2. Effect of IVIg on the expression of costimulatory molecules of DCs implicated in Treg expansion.** DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with IVIg for 24 hours. (A) The expression of PD-L1, PD-L2, OX-40L, and ICOSL depicted as percent positive cells (mean  $\pm$  SEM, n = 7-8) and (B) mean fluorescence intensity (MFI). The difference in expression levels of costimulatory molecules between Ctr and IVIg groups was not statistically significant.

Together, these results indicated that costimulatory molecules on DCs are not implicated in IVIg-mediated Treg expansion.

**IVIg induces COX-2 and PGE<sub>2</sub> in DCs**

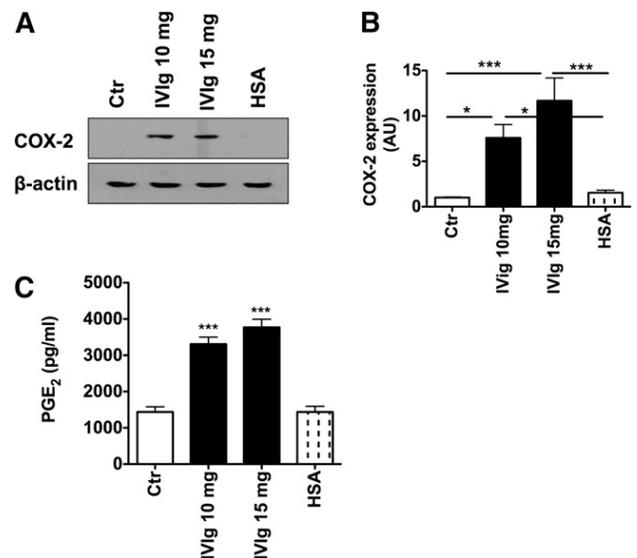
COX-2 and its product, PGE<sub>2</sub>, had been implicated in enhancing the attraction of Tregs and as a consequence for promoting the interaction of DCs and Tregs. In addition, COX-2 and PGE<sub>2</sub> were also shown to augment DC-mediated Treg expansion.<sup>37</sup> Therefore, to investigate whether expansion of Tregs by IVIg-treated DCs implicates the COX-2-PGE<sub>2</sub> pathway, DC lysates were subjected to western blot for analyzing the effect of IVIg on COX-2 expression. Untreated DCs showed minimal expression of COX-2 and its expression was not altered by HSA (Figure 3A-B). However, treatment of DCs with IVIg lead to significantly enhanced expression of COX-2 (Figure 3A-B).

To confirm whether high expression of COX-2 in IVIg-treated DCs resulted in PGE<sub>2</sub> production, the cell culture supernatants from IVIg-treated DCs were subjected to PGE<sub>2</sub> analysis. We found that IVIg-treated DCs produced significantly higher quantities of PGE<sub>2</sub> compared with untreated DCs and HSA-treated DCs (Figure 3C). The effect of IVIg on PGE<sub>2</sub> production was dose dependent. Further, increased PGE<sub>2</sub> observed in IVIg-treated DC cultures was a direct consequence of modulation of DCs and their intracellular signaling pathways and not due to soluble PGE<sub>2</sub> present in the Ig preparations. In fact, IVIg preparation was negative for PGE<sub>2</sub> as analyzed by ELISA.

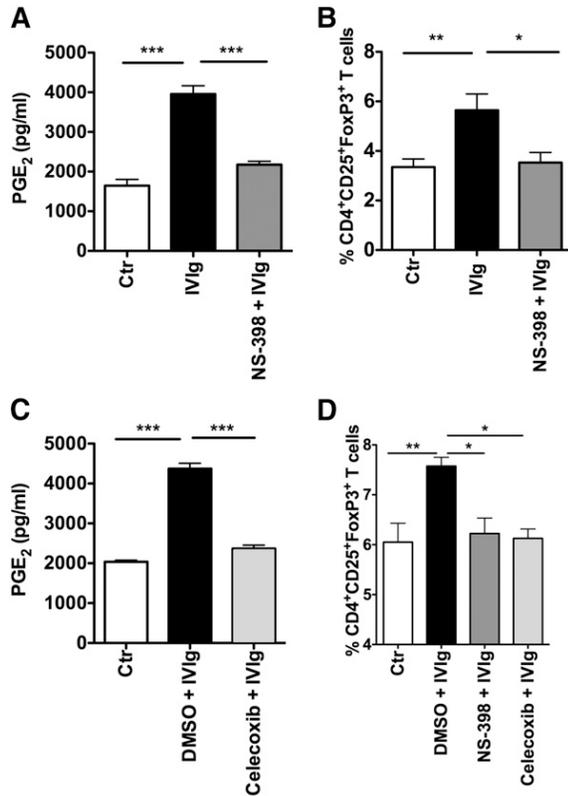
**Inhibition of COX-2 activity in DCs prevents IVIg-mediated Treg expansion**

To validate the role of COX-2-induced PGE<sub>2</sub> in IVIg-mediated Treg expansion, DCs were treated with COX-2 pharmacological inhibitors

NS-398 or celecoxib, followed by culture with IVIg. Inhibition of COX-2 activity resulted in a significant reduction in IVIg-induced PGE<sub>2</sub> production in DCs (Figure 4A,C), whereas DMSO, used as a solvent control, did not inhibit IVIg-mediated PGE<sub>2</sub> production.



**Figure 3. IVIg induces COX-2 and PGE<sub>2</sub> in DCs.** (A) DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with IVIg (10 mg or 15 mg) or HSA for 24 hours. Expression of COX-2 in DCs was analyzed by western blot. Representative blot of 7 independent experiments is shown. (B) The fold changes in COX-2 expression based on densitometry analysis of western blots (mean  $\pm$  SEM, n = 7). AU, arbitrary units. (C) The level (mean  $\pm$  SEM, n = 7) of production of PGE<sub>2</sub> (pg/mL) by DCs under the experimental conditions as explained above, \* P < .05; \*\*\*P < .001.



**Figure 4. Inhibition of COX-2 activity in DCs prevents IVIg-mediated Treg expansion.** (A) Amount of secretion of PGE<sub>2</sub> by DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with IVIg for 24 hours. In parallel, DCs were treated with COX-2 inhibitor NS-398 followed by treatment with IVIg (NS-398+IVIg). PGE<sub>2</sub> in cell-free culture supernatants was measured by ELISA (mean ± SEM, n = 7). (B) Percentage (mean ± SEM, n = 7) of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in the DC-CD4<sup>+</sup> T cell cocultures. DCs were treated as explained above. These DCs were washed extensively and cocultured with CD4<sup>+</sup> T cells for 4 days. Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) were analyzed by flow cytometry. (C) DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with DMSO (solvent control) followed by IVIg or with COX-2 inhibitor celecoxib followed by IVIg for 24 hours. The amount of secretion of PGE<sub>2</sub> by DCs was measured (mean ± SEM, n = 7). (D) Percentage (mean ± SEM, n = 4) of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in the DC-CD4<sup>+</sup> T cell cocultures. DCs were pretreated with DMSO, NS-398, or celecoxib followed by IVIg and then cocultured with CD4<sup>+</sup> T cells. \**P* < .05; \*\**P* < .01; \*\*\**P* < .001.

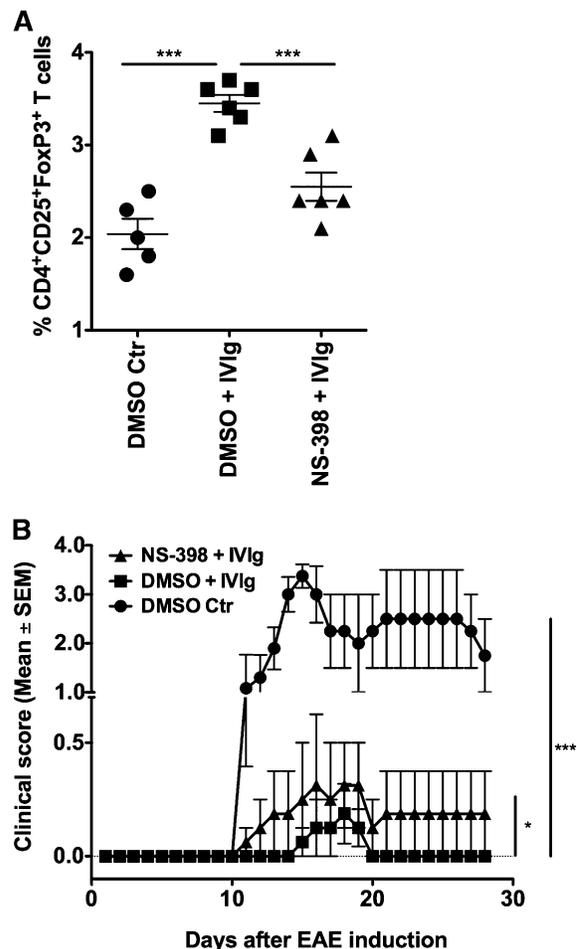
Further, COX-2 inhibition also abolished the ability of IVIg-treated DCs to expand Tregs (Figure 4B,D). Together, these results demonstrated that the COX-2-PGE<sub>2</sub> pathway is the critical player in IVIg-mediated expansion of Tregs.

#### Inhibition of COX-2 prevents IVIg-mediated Treg expansion in vivo in the EAE model

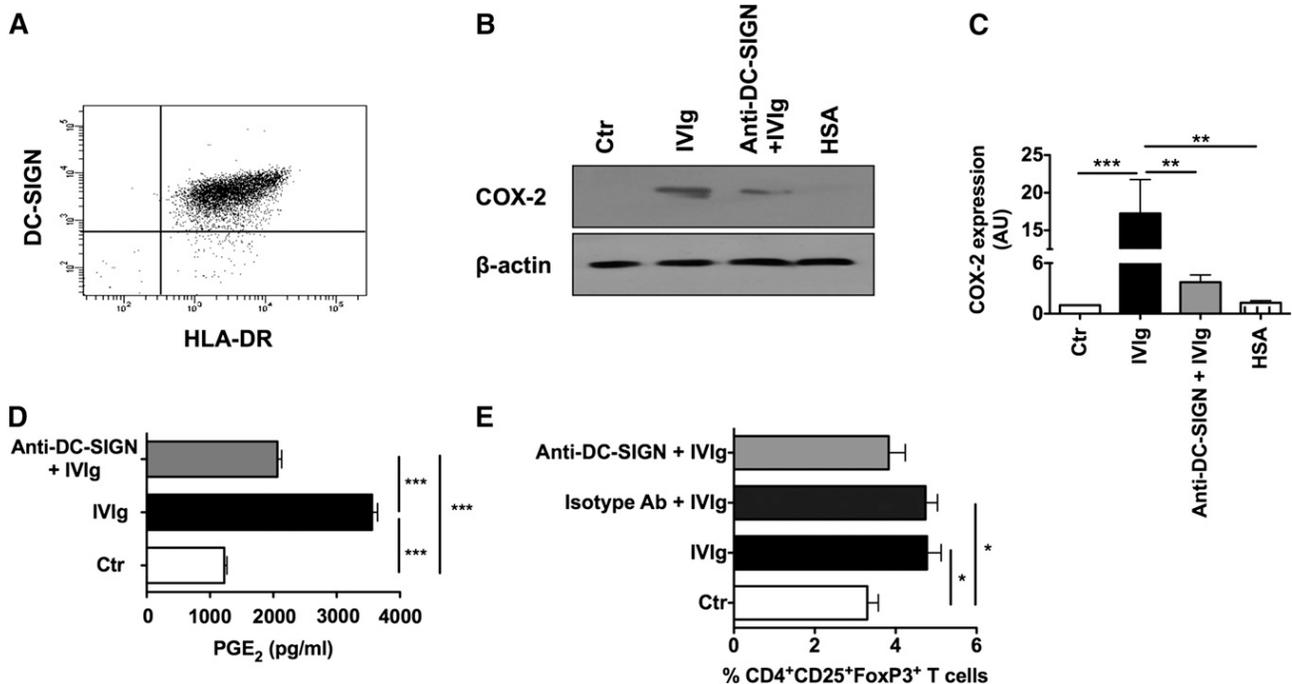
To confirm the role of COX-2 in IVIg-mediated Treg expansion in vivo, we resorted to the EAE model. Previous studies have demonstrated that IVIg protects mice from EAE and was associated with an expansion of Tregs.<sup>15,26</sup> Twelve days following the induction of EAE that corresponded to the onset of disease in the control group, mice were sacrificed and analyzed for Tregs in the spleen. In line with previous reports, we confirm that IVIg therapy in EAE is associated with significant expansion of Tregs (Figure 5A). Interestingly, COX-2 inhibitor NS-398 significantly prevented IVIg-mediated Treg expansion (Figure 5A), thus validating the in vivo relevance of COX-2-PGE<sub>2</sub> pathway in the IVIg-mediated Treg expansion.

We further found that modulation of IVIg-mediated Treg expansion by COX-2 inhibitor in vivo also had repercussions on the

evolution of EAE. As shown in Figure 5B, mice treated with combination of NS-398 and IVIg exhibited clear clinical signs of EAE in the early phase. In this group, clinical signs appeared as early as day 11 following immunization with MOG, whereas in the mice that received only IVIg, clinical signs began to appear on day 15. Thus, in mice that received NS-398 and IVIg, the clinical signs of EAE appeared 4 days earlier than in animals that received only IVIg. Also, the severity of disease was significantly higher in the group that received the NS-398 and IVIg combination compared with the group that was treated with only IVIg. Essentially, the pattern of evolution of the disease in mice that were treated with NS-398 and IVIg was similar to that of the control group. Further, the course of EAE was shorter in the mice that received only IVIg and after 5 days, these mice entered complete remission. On the contrary, mice that received the NS-398 and IVIg combination displayed a prolonged period of disease, ie, 18 days and diseased mice did not enter into remission (Figure 5B).



**Figure 5. Inhibition of COX-2 prevents IVIg-mediated Treg expansion and protection in vivo in EAE model.** EAE was induced in 10-week-old female C57BL/6J mice in 3 different groups. The first group received DMSO (solvent control for NS-398) on every alternative day until peak of the disease (day 16). The second group received IVIg (16 mg/mouse) every day and DMSO on every alternative day until day 16. The third group received IVIg every day and NS-398 (100 μg/mouse) on every alternative day until peak of the disease. (A) Mice were sacrificed on the day of onset of clinical signs (day 12) and splenic Tregs (CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup>) were analyzed by flow cytometry, \*\*\**P* < .001. (B) Repercussion of COX-2 inhibition in vivo on IVIg-mediated protection from EAE. The development of clinical signs in all the 3 groups of mice was followed until day 28 following induction of EAE, \**P* < .05; \*\*\**P* < .001.



**Figure 6. DC-SIGN is partially implicated in IVIg-mediated COX-2 expression and PGE<sub>2</sub> production in DCs and Treg expansion.** (A) Expression of DC-SIGN in DCs as analyzed by flow cytometry. (B) Western-blot analysis of COX-2 expression in DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with IVIg or HSA for 24 hours. In parallel, DCs were preincubated with blocking antibodies to DC-SIGN followed by treatment with IVIg (Anti-DC-SIGN+IVIg). Representative blot of 6 experiments is shown. (C) The fold changes in the COX-2 expression based on densitometry analysis of western blots (mean  $\pm$  SEM, n = 6). AU, arbitrary units. (D) Secretion (mean  $\pm$  SEM, n = 6) of PGE<sub>2</sub> by DCs that were treated as explained above. (E) Percentage (mean  $\pm$  SEM, n = 7) of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells in the DC-CD4<sup>+</sup> T cell cocultures. DCs were pretreated with DC-SIGN blocking antibodies or isotype control antibodies followed by treatment with IVIg and then cocultured with CD4<sup>+</sup> T cells, \**P* < .05; \*\**P* < .01; \*\*\**P* < .001.

#### DC-SIGN is partially implicated in IVIg-mediated PGE<sub>2</sub> production in DCs

Recently, it was demonstrated that the antiinflammatory functions of IVIg are mediated via interaction of terminal  $\alpha$ 2,6-sialic acid linkages at Asp297 of IVIg with C-type lectin receptor SIGN-R1 (in mice) or DC-SIGN (in humans),<sup>38-40</sup> although antibody-mediated experimental models of ITP show that the protective effect of IVIg is independent of sialylation of IgG.<sup>41</sup> Therefore, we investigated whether the interaction of DC-SIGN and IVIg is indispensable for the expression of COX-2 and production of PGE<sub>2</sub> by DCs. We confirm that the monocyte-derived human DCs express high levels of DC-SIGN (Figure 6A). Further, blockade of DC-SIGN on DCs before IVIg treatment lead to significant inhibition of IVIg-mediated COX-2 expression (Figure 6B-C) and of PGE<sub>2</sub> production (Figure 6D). However, DC-SIGN blockade leads only to partial (*P* > .05) inhibition of Treg expansion by IVIg (Figure 6E). These results thus indicated that IVIg-mediated activation of COX-2-PGE<sub>2</sub> pathway is mediated at least in part by signaling via DC-SIGN. The non-significant inhibition of Treg expansion by IVIg-treated DCs upon DC-SIGN blockade despite significant inhibition of COX-2 and PGE<sub>2</sub> could be due to either receptor recycling on DCs or due to lack of total abolition of PGE<sub>2</sub> production in DCs upon DC-SIGN blockade.

#### IVIg-mediated COX-2 induction and PGE<sub>2</sub> production in DCs are F(ab')<sub>2</sub> dependent

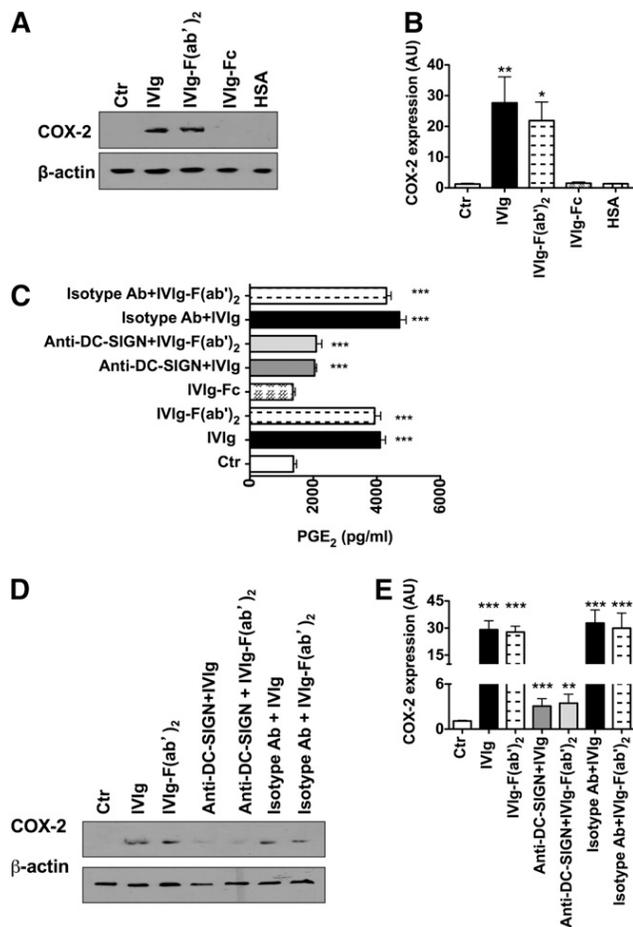
By using *Sambucus nigra* agglutinin lectin fractionation, 2 recent reports demonstrated that a significant amount of sialylation is found in the Fab region of IVIg and not in the Fc region.<sup>42,43</sup> Accordingly, the antiinflammatory activity was observed with Fab sialylation and

not with Fc. Interestingly, F(ab')<sub>2</sub> Fragments of IVIg exerted an antiinflammatory effect on DCs similar to that of intact IgG molecules of IVIg.<sup>29,42,44</sup> Therefore, we explored the role of F(ab')<sub>2</sub> and Fc fragments of IVIg on COX-2 activation and PGE<sub>2</sub> synthesis by DCs. We found that IVIg-mediated COX-2 induction and PGE<sub>2</sub> production in DCs were mediated mainly via F(ab')<sub>2</sub> fragments (Figure 7A-C), whereas Fc fragments had a minimal effect. Similar to results with IVIg, blockade of DC-SIGN on DCs lead to significant inhibition of COX-2 expression and PGE<sub>2</sub> production by F(ab')<sub>2</sub> fragments of IVIg (Figure 7C-E).

## Discussion

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs play a vital role in preventing inflammation and autoimmunity.<sup>14</sup> The deficiency of Tregs in humans as a consequence of mutations in the FoxP3 gene causes a severe autoimmune inflammatory disorder called immune dysregulation polyendocrinopathy enteropathy X-linked syndrome. Similarly, in experimental models, deficiency of Tregs either due to genetic knockdown of FoxP3 gene or depletion leads to either appearance of autoimmune disease or exacerbation of ongoing disease.<sup>14</sup> Therefore, therapeutic approaches that enhance Treg numbers and/or amelioration of their function have drawn considerable interest in recent years.

Several reports have demonstrated that IVIg therapy is associated with an expansion of Tregs in the periphery.<sup>15,17,18,21-23</sup> In the present study, we aimed at identifying the cellular and molecular mechanism by which IVIg expands Tregs. Among professional antigen-presenting cells, myeloid DCs were considered as potent immune cells that could efficiently expand Tregs.<sup>45</sup> In addition, ours and other reports have demonstrated that IVIg exerts modulatory



**Figure 7. IVIg-mediated COX-2 induction and PGE<sub>2</sub> production in DCs are F(ab)<sub>2</sub> dependent.** (A,D) Western-blot analysis of COX-2 expression in DCs. DCs were cultured in GM-CSF and IL-4 alone (Ctr) or with IVIg or equimolar concentrations of either F(ab)<sub>2</sub> fragments or Fc fragments of IVIg or HSA for 24 hours (A). In some experiments, DCs were preincubated with blocking antibodies to DC-SIGN or isotype control antibodies followed by treatment with equimolar concentrations of either IVIg or F(ab)<sub>2</sub> fragment of IVIg (D). Representative blot of 6 to 7 experiments is shown. (B,E) The fold changes in the COX-2 expression (mean ± SEM, n = 6-7) based on densitometry analysis of western blots in the above experiments. AU, arbitrary units. (C) Secretion (mean ± SEM, n = 7) of PGE<sub>2</sub> by DC that were treated as explained above, \*P < .05; \*\*P < .01; \*\*\*P < .001.

effect on DCs,<sup>29,30,32</sup> thus indicating that Treg expansion by IVIg observed in autoimmune patients and experimental models might be a repercussion of DC education by IVIg.

Available data indicate that DCs can expand Tregs via costimulatory molecules such as PD-L1 and 2, ICOSL, and OX-40L.<sup>33-36,46</sup> The interaction of PD-L1 and 2 with PD-1, the members of CD28/B7 family, has a critical role in balancing the T-cell tolerance through inhibiting the activation of effector cells while promoting the generation and expansion of Tregs.<sup>33,34</sup> Pathogens such as *Mycobacterium* can hijack the immune system by activating the PD-L-PD-1 pathway and thus enhancing the Tregs and preventing the protective immune responses.<sup>34,46</sup> Although ICOS, another member of CD28/B7 family of costimulatory molecules, and OX-40, a member of tumor necrosis factor receptor superfamily, are constitutively expressed on Tregs, their expression on other T cell subsets is restricted to activated cells.<sup>35,36</sup> In contrast to PD-1, these costimulatory molecules provide positive stimuli to activated CD4<sup>+</sup> T cells and depending on the context of the inflammatory responses, they can promote Th1, Th2, or Th17 responses.<sup>35,36</sup> Thus, the ability

of ICOSL and OX-40L to induce Treg activation and expansion depends on the cytokine milieu.<sup>35,36</sup> We found that IVIg did not enhance or modify the expression of any of the costimulatory molecules on DCs that are implicated in Treg expansion, including Notch ligands,<sup>47</sup> thus indicating that costimulatory pathway does not play an important role in IVIg-mediated Treg expansion.

Next, we turned our attention toward DC-derived soluble factors such as TGF-β and PGE<sub>2</sub> that are known to provide stimulation for Treg induction and expansion.<sup>45,48,49</sup> However, we previously observed that in the presence of TGF-β, there was no induction of human Tregs by IVIg<sup>20</sup> and TGF-β was not increased in the splenocytes of IVIg-treated mice.<sup>15</sup> This data suggested that TGF-β is not implicated in IVIg-mediated Treg expansion.

PGE<sub>2</sub> is an arachidonic acid metabolite product. COX enzymes play a critical role in converting arachidonic acid released from the cell membrane into prostaglandins. Among 3 COX isoenzymes, COX-1 is constitutively expressed in variety of cells and act as a housekeeping enzyme to maintain the basal levels of PGE<sub>2</sub>. On the contrary, COX-2 is an inducible enzyme.<sup>37</sup> As PGE<sub>2</sub> can bind to 4 different receptors, PGE<sub>2</sub> exerts diverse biological functions on the immune cells. These functions include regulation of T-cell differentiation and proliferation, inhibition of cytokines and chemokines by innate immune cells such as DCs and macrophages and suppression of effector functions of NK cells and granulocytes. Thus, PGE<sub>2</sub> can regulate the inflammation and immune responses.<sup>37</sup>

Several lines of evidence also indicate that PGE<sub>2</sub> enhances the generation of Tregs and their expansion both in humans and mice.<sup>37</sup> In addition, PGE<sub>2</sub> also mediate suppressive functions of Tregs.<sup>49</sup> Vaccination with PGE<sub>2</sub>-educated DCs has been shown to induce Treg expansion in cancer patients.<sup>45</sup> Elevated levels of PGE<sub>2</sub> and COX-2 activity leading to induction of Tregs and FoxP3, expansion of Tregs, and enhanced suppressive functions have been described in tumor conditions.<sup>50</sup> Thus, COX2-PGE<sub>2</sub>-Treg represents an immunosuppressive network. As COX-2 inhibition in DCs lead to downregulation of PGE<sub>2</sub> and IVIg-mediated Treg expansion and inhibition of COX-2 in vivo has been shown to reduce the frequency of Tregs and their activity,<sup>50</sup> we might conclude that Treg expansion in vivo in autoimmune patients following IVIg therapy might be due to enhancement of PGE<sub>2</sub>. In fact, inhibition of COX-2 activity by systemic injection of NS-398 leads to significant downregulation of IVIg-mediated Treg expansion in the EAE model. Also, COX-2 inhibitor significantly decreased IVIg-mediated protection from EAE. In line with these results, our preliminary data also suggest that beneficial effects of IVIg in autoimmune patients are associated with an enhancement of PGE<sub>2</sub> in the circulation.

Our results thus provide a novel cellular and molecular mechanism for IVIg in that it links innate and adaptive immune compartment to mediate immune tolerance via Tregs. As IVIg-treated circulating monocytes did not expand Tregs imply that IVIg-mediated Treg expansion could be specific for DCs. Further work is necessary to identify DC subset(s) implicated in COX-2-dependent IVIg-mediated Treg expansion. Our results also indicate that Treg expansion by IVIg in autoimmune patients could involve a 2-step process: recognition and signaling in DCs by IVIg leading to COX-2 activation and PGE<sub>2</sub> synthesis and action of PGE<sub>2</sub> on Tregs leading to their expansion.

Various animal models implicate a role for sialylation of Fc fragments in the beneficial effect of IVIg.<sup>38-40</sup> However, recent publications also demonstrate that Fc-sialylation is dispensable for antiinflammatory effects of IVIg.<sup>29,41,42,44</sup> We have observed that IVIg-mediated COX-2 induction is F(ab)<sub>2</sub> dependent in part via DC-SIGN, indicating that sialylation in F(ab)<sub>2</sub> fragments does have a role in the observed phenomenon. That DC-SIGN blockade did not

completely abolish PGE<sub>2</sub> production and Treg expansion by IVIg also points toward a sialylation/DC-SIGN-independent mechanism for IVIg on DCs to mediate Treg expansion. Although certain diseases such as ITP have been demonstrated to benefit from Fc-fragments of IVIg, we believe that bifurcation of IVIg functions as Fc- or F(ab')<sub>2</sub>-dependent is artificial; no single mechanism of IVIg appears to be solely responsible for the beneficial effects of IVIg observed in diverse autoimmune diseases.

## Acknowledgments

The authors thank Chaitrali Saha for the help.

The work was supported by Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, Université Paris Descartes (J.B. and S.V.K.); Centre National de la Recherche Scientifique (S.V.K.); European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement HEALTH-2010.2.4.5-2 ALLFUN (J.B.); Indian Institute of Science, Department of Biotechnology, Department of Science and Technology, Council for Scientific and Industrial Research, India (K.N.B.);

Coopération INSERM-ICMR-AO 2009/2010 (J.B. and K.N.B.); fellowships from INSERM-ICMR, Council for Scientific and Industrial Research (J.T.); and Journées de Neurologie de Langue Française (M.R.).

## Authorship

Contribution: J.T., P.H., M.S., and M.S.M. performed the experiments and analyzed the data; M.R. performed the experiments; J.-M.V. and L.M. provided advice; K.N.B. and S.V.K. provided advice and analyzed the data; and J.B. conceived the project, designed the experiments, analyzed the data and wrote the paper

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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## **Intravenous Gammaglobulin Inhibits Encephalitogenic Potential of Pathogenic T Cells and Interferes with their Trafficking to the Central Nervous System, Implicating S phingosine-1 Phosphate Receptor 1– Mammalian Target of Rapamycin Axis**

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*J Immunol* 2013; 190:4535-4541; Prepublished online 22  
March 2013;

doi: 10.4049/jimmunol.1201965

<http://www.jimmunol.org/content/190/9/4535>

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- Supplementary  
Material** <http://www.jimmunol.org/content/suppl/2013/03/22/jimmunol.120196.5.DC1.html>
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Intravenous Gammaglobulin Inhibits Encephalitogenic Potential of Pathogenic T Cells and Interferes with their Trafficking to the Central Nervous System, Implicating Sphingosine-1 Phosphate Receptor 1—Mammalian Target of Rapamycin Axis

Shivashankar Othy,<sup>\*,†</sup> Pushpa Hegde,<sup>\*,‡</sup> Selma Topçu,<sup>\*,†</sup> Meenu Sharma,<sup>\*,‡</sup> Mohan S. Maddur,<sup>\*,†</sup> Sebastien Lacroix-Desmazes,<sup>\*,†,§,¶</sup> Jagadeesh Bayry,<sup>\*,†,§,¶</sup> and Srinivasa V. Kaveri<sup>\*,†,§,¶</sup>

Despite an increasing use of high-dose therapy of i.v. gammaglobulin (IVIg) in the treatment of various T cell- and Ab-mediated inflammatory and autoimmune diseases, comprehension of the mechanisms underlying its therapeutic benefit has remained a major challenge. Particularly, the effect of IVIg in T cell-mediated autoimmune conditions remains unexplored. Using an actively induced experimental autoimmune encephalomyelitis model, a T cell-mediated autoimmune condition, we demonstrate that IVIg inhibits the differentiation of naive CD4 T cells into encephalitogenic subsets (Th1 and Th17 cells) and concomitantly induces an expansion of Foxp3<sup>+</sup> regulatory T cells. Further, IVIg renders effector T cells less pathogenic by decreasing the expression of encephalitogenic molecular players like GM-CSF and podoplanin. Intriguingly and contrary to the current arguments, the inhibitory FcγRIIB is dispensable for IVIg-mediated reciprocal modulation of effector and regulatory CD4 subsets. Additionally, F(ab')<sub>2</sub> fragments also retained this function of IVIg. IVIg or F(ab')<sub>2</sub> fragments decrease the sphingosine-1 phosphate receptor on CD4 cells, thus sequestering these cells in the draining lymph nodes and decreasing their infiltration into the CNS. Our study reveals a novel role of Igs in the modulation of polarization and trafficking of T lymphocytes, accounting for the observed beneficial effect in IVIg therapy. *The Journal of Immunology*, 2013, 190: 4535–4541.

**A**ctivation of naive CD4 T cells and their differentiation into various subsets is the crucial event in the initiation of an adaptive immune response. Local cytokine milieu and complex signaling events at the immune synapse between the Ag-specific Th cell and APC determine the fate of these cells. For example, in the presence of cytokines IL-12 and IFN-γ, naive Th cells are polarized toward Th1, IL-4 skews them to the Th2 phenotype,

IL-6 and TGF-β polarize CD4 T cells to Th17, and TGF-β alone is involved in induction of regulatory T cells (Treg) (1). Dysregulated innate and adaptive immune cells lead to undesired pathogenic immune response to self-Ags and inflammatory pathologies. Several lines of evidence suggest that Th1 and Th17 play critical role in the initiation and progression of many autoimmune and inflammatory diseases. Cytokines produced by Th17 cells such as IL-17A, IL-17F, IL-21, IL-22, and GM-CSF are involved in recruitment of other inflammatory and effector immune cells at the site of inflammation, thus leading to tissue destruction (2, 3). Th17 are one of the major pathogenic cells in immune-mediated tissue damage, such as multiple sclerosis, chronic inflammatory bowel disease, psoriasis, systemic lupus erythematosus, asthma, allergic contact dermatitis, dermatomyositis, pemphigus, allergic rhinitis, anti-neutrophil cytoplasmic Ab-associated vasculitis, and rheumatoid arthritis (4).

In contrast, Treg expressing the transcription factor Foxp3 are involved in the suppression of autoreactive T cells and regulation of immune response. Treg actively regulate autoimmunity throughout the lifespan and are indispensable for maintenance of immune homeostasis (5). Treg inhibit the proliferation and cytokine production by conventional T cells and can also regulate the functions of NK cells and professional APC. The suppression of immune responses by Treg generally requires direct cell–cell contact, implicating CTLA-4, CD39, and LAG-3, but soluble factors, particularly TGF-β and IL-10, have also been implicated (6). Several autoimmune diseases are associated with reduced number of Treg or defects in their functions. The balance between Treg and effector T (T<sub>eff</sub>) cells, including Th17 and Th1, determines the course of immune-mediated disorders.

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Received for publication July 18, 2012. Accepted for publication February 22, 2013.

This work was supported by grants from INSERM, Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Université Paris Descartes, and Talents Program, Talecris Biotherapeutics. S.O. received a fellowship for a Ph.D. from the Erasmus Mundus Program.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; IVIg, i.v. gammaglobulin; mTOR, mammalian target of rapamycin; S1P, sphingosine-1 phosphate; S1P1, sphingosine-1 phosphate receptor 1; T<sub>eff</sub>, effector T; Treg, regulatory T cell; WT, wild-type.

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High-dose therapy of i.v. gammaglobulin (IVIg) is being widely used to treat various autoimmune and inflammatory conditions. IVIg is a therapeutic preparation of normal human polyclonal IgG obtained from pools of plasma from a large number of healthy blood donors. Understanding the cellular and molecular mechanisms by which IVIg exerts its anti-inflammatory effects in highly diverse pathological situations incriminating autoantibodies, pathogenic T cells, complement-mediated tissue damage, or dysregulated cytokine network has rendered the area particularly challenging. IVIg exerts its beneficial effects by several mutually nonexclusive mechanisms (7–9). Many of these effects are deduced based on animal models of Ab-mediated autoimmunity and *ex vivo* studies (10). However, therapeutic benefit of IVIg is also clearly established in several T cell-mediated autoimmune pathologies (11–14). Previous reports from our laboratory show that the beneficial effect of IVIg in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis and a T cell-mediated pathology, is associated with a peripheral expansion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg and a significant amelioration of their functions (15). The emerging knowledge that Th17 cells as potent pathogenic T cells in autoimmunity and that Th17 and Treg exercise reciprocal regulatory effects (16) have raised additional interesting possibilities on the mechanisms of IVIg.

We surmised that IVIg restores the dysregulated equilibrium between Th17 cells and Treg in T cell-mediated pathologies. In the current study, using actively induced EAE, we thus set out to investigate whether IVIg modulates T cell polarization *in vivo* and tilts the balance from pathogenic T<sub>eff</sub> cells toward Treg. We found that IVIg exerted its beneficial effect in EAE by inhibiting the differentiation of Th17 and Th1 cells and simultaneously increasing Treg. Interestingly, FcγRIIB was dispensable for this effect, and F(ab')<sub>2</sub> fragments of IVIg recapitulated the effect of intact IgG molecules in reciprocally modulating T<sub>eff</sub> cells and Treg. Further, IVIg inhibited the encephalitogenic potential of pathogenic T cells and interfered with their trafficking to the target organ implicating sphingosine-1 phosphate receptor 1 (S1P1)—mammalian target of rapamycin (mTOR) axis.

## Materials and Methods

### Animals

All animal studies were performed according to the guidelines of Charles Darwin ethical committee for animal experimentation (Université Pierre et Marie Curie Paris) at the pathogen-free animal facility of Cordelier Research Center, Paris. Wild-type (WT) C57BL/6J mice were purchased from Janvier Laboratories, and FcγRIIB<sup>-/-</sup> mice (8 wk old) on the C57BL/6 background were obtained from Taconic Farms.

### Induction of EAE, assessment, and IVIg treatment

EAE was induced in 10-wk-old female C57BL/6J or FcγRIIB<sup>-/-</sup> mice as previously reported (15). Briefly, 200 μl emulsion was injected s.c. at two sites over the flank region. Each mouse received 200 μg MOG<sub>35–55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK; PolyPeptide Laboratory, Strasbourg, France) emulsified in CFA (Sigma-Aldrich) containing 880 μg nonviable *Mycobacterium tuberculosis* H37RA (Difco Laboratories). Additionally, 300 ng pertussis toxin (List Biologic Laboratories) was injected i.v. on the day of immunization and 48 h later. Mice were daily assessed for the development of clinical signs according to the following scoring pattern: 0, no signs; 1, tail paresis; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; and 5, moribund. IVIg (Gamunex 10% w/v; Talecris Biotherapeutics) was given at 0.8 g/kg i.p. from the day of the immunization until the peak of the disease (days 16–18). Mice in F(ab')<sub>2</sub> group received 0.55 g/kg body weight (equimolar concentration). Control mice received equal volumes 0.2 M glycine (excipient used in Gamunex).

### Generation of F(ab')<sub>2</sub> fragments

F(ab')<sub>2</sub> fragments were generated by digesting IVIg with pepsin (Sigma Aldrich) at 50:1 ratio for 18 h in 0.2 M sodium acetate buffer (pH 4.1).

F(ab')<sub>2</sub> was extensively dialyzed against sterile PBS and filtered through a 0.22-μm membrane before injecting to the mice. Purity of F(ab')<sub>2</sub> was verified by SDS-PAGE and Coomassie blue staining.

### Isolation of cells from blood, spleen, draining lymph nodes, and CNS

On the day of the sacrifice, blood was collected from mice under xylazine/ketamine anesthesia. Mice were perfused with 40 ml of 0.2 mM EDTA in PBS through the left ventricle and spleen, draining lymph nodes (DLN; inguinal), brain, and spinal cord were collected. Single-cell suspensions were obtained by mechanical disaggregation and passing the cells through a 70-μm nylon membrane filter. Mononuclear cells from CNS were isolated using a 37.5% Percoll gradient centrifugation (GE Healthcare). RBCs were lysed using ACK lysis buffer.

### Flow cytometry

To detect the intracellular cytokines, 1.5 × 10<sup>6</sup> cells were stimulated with 25 ng PMA and 1 μg ionomycin (Sigma-Aldrich) in 10% FCS/RPMI 1640 for 4 h at 37°C. Monensin (GolgiStop; BD Biosciences) was added according to the manufacturer's instructions to block the protein transport. Cells were surface labeled with anti-mouse CD4-Pacific Blue (clone RM4-5; BD Biosciences), podoplanin-PE (clone 8.1.1; BioLegend), and S1P1-allophycocyanin (R&D Systems) Ab after the blocking FcRs with anti-mouse CD16/32 Ab (BD Fc Block). Surface-stained cells were washed, fixed, and permeabilized using a Foxp3 staining buffer set (eBioscience). Abs to detect intracellular cytokines, IL-17A-A488 (clone 11B11; BD Biosciences), IFN-γ-APC (clone XMG1.2; BD Biosciences), Foxp3-PE (clone FJK16s; eBioscience), GM-CSF-PE (clone MP1-22E9; eBioscience), CD11b-A700 (eBioscience), GLAST-1-PE (Miltenyi Biotec), and phospho-S6-A488 (Cell Signaling Technology), were used in permeabilization buffer (eBioscience). Cells were acquired and analyzed using a BD LSR II and FACS Diva software (BD Biosciences).

### Ex vivo stimulation and cytokines assays

A single-cell suspension was prepared from DLN, and the spleen was stimulated with 10 μg/ml MOG<sub>35–55</sub> for 24 h in RPMI 1640 culture medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 10% FCS. Cytokine concentration in the supernatant and serum was estimated by using cytometric bead array (Mouse Th1/Th2/Th17 cytokine CBA kit; BD Biosciences).

### Statistical analysis

Two-way ANOVA with Bonferroni's posttest was used to analyze daily clinical score. Mann-Whitney's *U* test was used to compare parameters between control and IVIg group. The *p* values obtained are indicated in the figure legends.

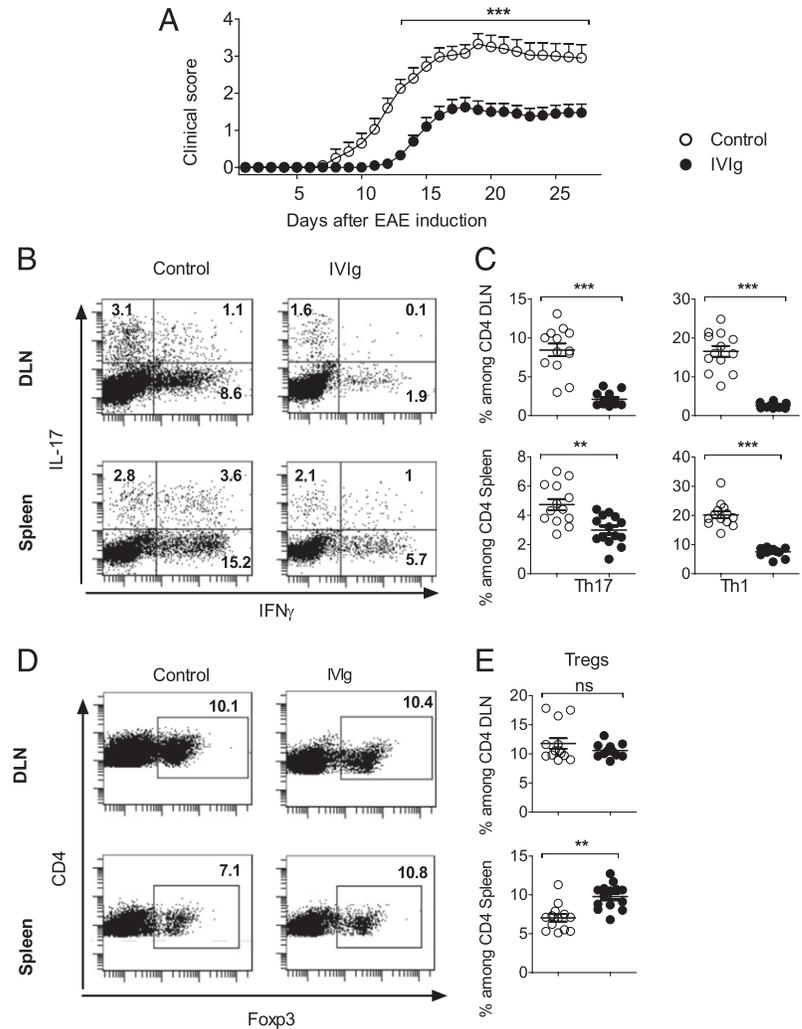
## Results

### IVIg delays the onset of EAE and decreases severity of the disease by inhibiting Th17 and Th1 cells and increasing Treg

To understand the modulation of effector and regulatory CD4 subsets by IVIg in an autoimmune setup, EAE was induced in WT C57BL/6J mice using MOG<sub>35–55</sub> emulsified in CFA. From the day of immunization to the peak of the disease (days 16–18), mice in the control and IVIg group were treated with 0.2 M glycine and IVIg (Gamunex), respectively. Control mice started to display clinical signs from day 7, and mean score at the peak was 3.5. IVIg significantly delayed the onset of EAE (day 11) and decreased severity of the disease (Fig. 1A). Because there is the possibility of a xenoantigen-specific response to human IgG, and because it is impractical to obtain large amounts of polyclonal, polyspecific mice IgG of a diverse repertoire, we have demonstrated that equimolar concentrations of human serum albumin in rat and mouse systems are unable to display any protective effects in EAE (15, 17).

MOG<sub>35–55</sub>-specific naive CD4 cells differentiating into Th17 and Th1 cells are known to be the pathogenic in EAE (18). To investigate whether IVIg affects differentiation of Th17 and Th1 cells in EAE, mice in each group were sacrificed 9 d after EAE induction (onset) and analyzed for their signature cytokines (IL-17 in Th17 and IFN-γ in Th1) by flow cytometry. We observed robust

**FIGURE 1.** IVIg protects mice against EAE by decreasing Th17 and Th1 cells and enhancing CD4 Foxp3 Treg. **(A)** IVIg delays the onset and decreases severity of EAE. EAE was induced in 10–12-wk-old female WT C57BL/6J mice. From days 0–18, mice in control (open circles) and IVIg (filled circles) groups received i.p. injections of 0.2 M glycine and IVIg (0.8 g/kg Gamunex), respectively. Development of clinical signs was monitored daily as described in *Materials and Methods*. Mean clinical scores from two independent experiments are presented ( $n = 20$ ). Error bars represent SEM. Significance of difference in the EAE scores between the control and IVIg groups was analyzed by the two-way ANOVA with Bonferroni post  $t$  test.  $***p < 0.001$ . **(B)** Mice were sacrificed on the day of onset (day 9). DLN and spleen were obtained for flow cytometry. Representative dot plots showing CD4 cells that are positive for IL-17 (y-axis) and IFN- $\gamma$  (x-axis). Number in each quadrant represents the percent of cells among CD4 population. DLN (inguinal, upper panel) and spleen (bottom panel) from control (left panel) and IVIg (right panel) mice are shown. **(C)** Plots show frequency of Th17 (left panel) and Th1 (right panel) from DLN (upper panel) and spleen (bottom panel). **(D)** Representative dot plots showing CD4 (y-axis) and Foxp3 (x-axis) from control (left panel) and IVIg group (right panel) of same mice shown in (B). Number in each quadrant represents the percent of Foxp3<sup>+</sup> cells among CD4 population. Data from DLN (upper panel) and spleen (bottom panel) are represented. **(E)** Frequency of CD4<sup>+</sup>Foxp3<sup>+</sup> in DLN (top panel) and in spleen (bottom panel) from two independent experiments are shown. (C and E) Open circles represent control mice, and filled circles represent IVIg-treated mice. Data are obtained from two independent experiments ( $n > 12$ ). Mean value is depicted as a horizontal line among the symbols and error bars represent SEM.  $**p < 0.01$ ,  $***p < 0.001$  determined by Mann–Whitney  $U$  test.



decrease in Th17 and Th1 cells in inguinal DLN of IVIg-treated mice (Fig. 1B, 1C, top panel). A similar trend was also observed in spleens of IVIg mice (Fig. 1B, 1C, bottom panel). Inhibition of Th17 and Th1 cells by IVIg in vivo was further confirmed by the profile of cytokine secretion. Cells from DLN and spleen on day 9 were stimulated ex vivo with MOG<sub>35–55</sub> for 24 h, and cell-free supernatants were analyzed for cytokines. Cells from IVIg-treated mice secreted decreased amounts of IL-17 and IFN- $\gamma$  (Supplemental Fig. 1A, 1B) as compared with control. IVIg did not affect the CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in DLN (Fig. 1D, 1E, top panel). We observed a concomitant increase in CD4<sup>+</sup>Foxp3<sup>+</sup> Treg in the spleens of IVIg-treated mice (Fig. 1D, 1E, bottom panel). Thus, IVIg reciprocally modulates T<sub>eff</sub> and Treg in EAE.

#### IVIg decreases pathogenicity of T cells by decreasing the expression of encephalitogenic molecular players like GM-CSF and podoplanin

Both Th17 and Th1 cells are involved in EAE; however, Th17 cells are considered the main pathogenic mediators (19). Interestingly, signature cytokines of Th17 cells like IL-17A, IL-17F, IL-21, and IL-22 are dispensable for the induction of EAE (20). Encephalitogenic potential of Th17 cells in EAE has been attributed to molecules like GM-CSF (2) and podoplanin (21). Hence, we set out to determine whether IVIg affects these key molecules. Injected to EAE mice, IVIg significantly decreased CD4<sup>+</sup>GM-CSF<sup>+</sup> cells in DLN (Fig. 2A, 2B). Additionally, there was a decrease in the absolute number of these cells in CNS (Fig. 2C). Similarly, IVIg also

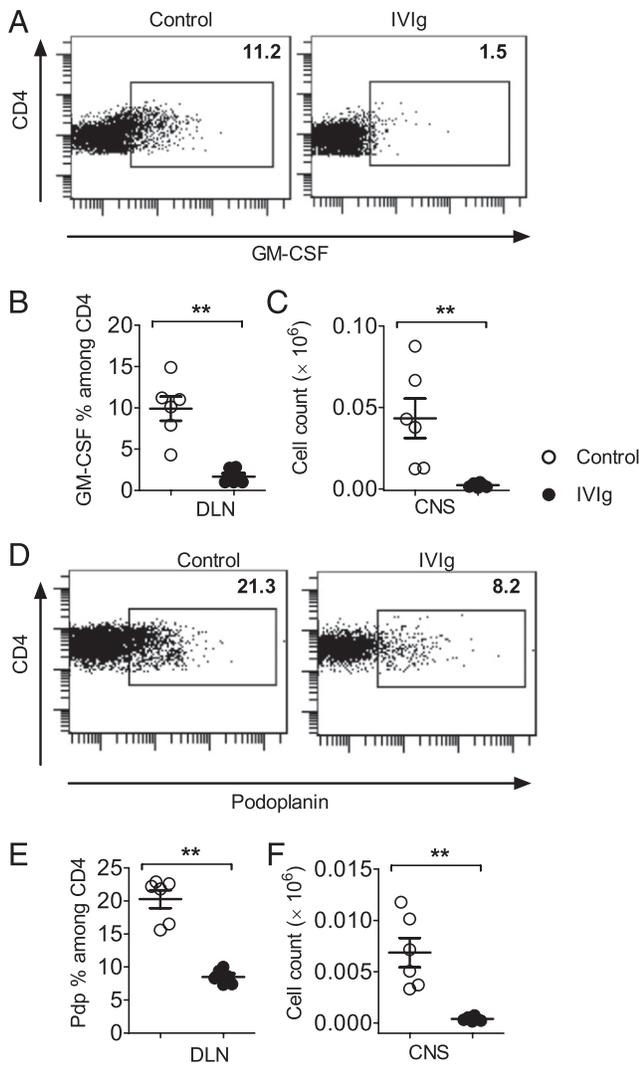
downregulated the expression of podoplanin on CD4 cells in EAE (Fig. 2D–F). Thus, in addition to inhibiting the differentiation of Th17 cells, by downregulating GM-CSF and PdP, IVIg renders these cells less encephalitogenic.

#### Fc $\gamma$ RIIB is dispensable for IVIg-mediated reciprocal modulation of effector and regulatory CD4 subsets

Anti-inflammatory effect of IVIg in several animal models of Ab-mediated pathology is attributed to the inhibitory Fc receptor Fc $\gamma$ RIIB (22, 23). The role of Fc $\gamma$ RIIB in protection of EAE mice by IVIg is, however, unexplored. To examine whether reciprocal modulation of CD4 subsets by IVIg in EAE is dependent on Fc $\gamma$ RIIB, we induced EAE in Fc $\gamma$ RIIB<sup>-/-</sup> mice under C57BL/6J background and analyzed various CD4 subsets 9 d after immunization. IVIg inhibited Th17 and Th1 cells in Fc $\gamma$ RIIB<sup>-/-</sup>-EAE mice (Fig. 3A, 3B). Concomitantly, we observed an increase in the percent of Treg in the spleen (Fig. 3B). Further, IVIg treatment decreased the expression of GM-CSF and podoplanin in Fc $\gamma$ RIIB<sup>-/-</sup> mice, rendering them less encephalitogenic (Fig. 3C, 3D). Altogether, these data reveal that Fc $\gamma$ RIIB is dispensable for the reciprocal modulation of effector and regulatory CD4 subsets by IVIg in EAE.

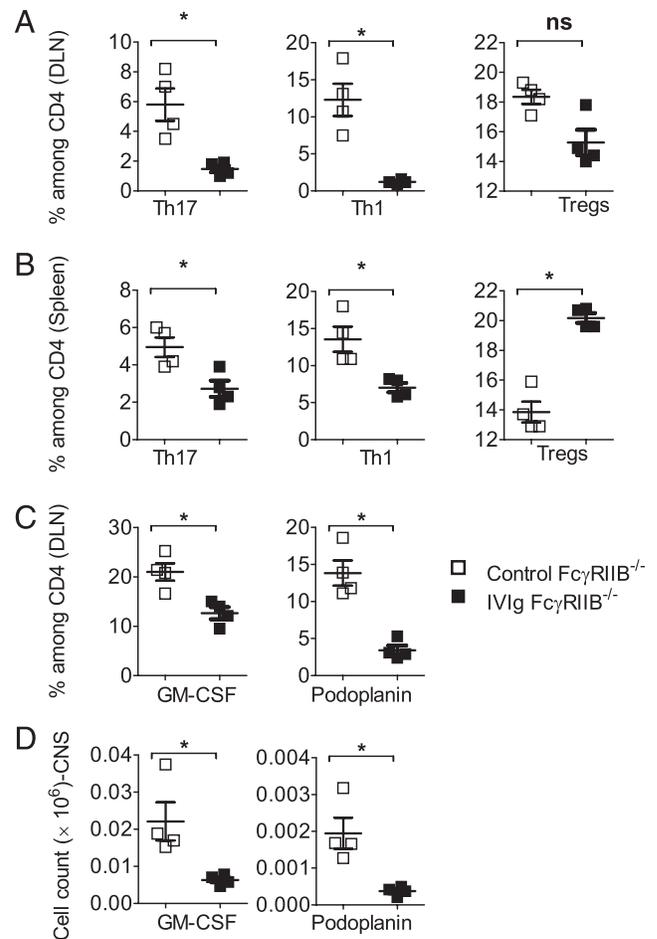
#### F(ab)<sub>2</sub> fragments recapitulate the capacity of IVIg to reciprocally modulate effector and regulatory CD4 subsets in EAE

As Fc part of IVIg is implicated in the Fc $\gamma$ RIIB-dependent functions of IVIg, our results on dispensability of Fc $\gamma$ RIIB in IVIg-



**FIGURE 2.** IVIg renders CD4 cells less pathogenic in EAE by down-regulating encephalitogenic molecules like GM-CSF and podoplanin. EAE was induced in WT C57BL/6J mice, and IVIg was injected daily. Nine days after induction, mice from each group were sacrificed, and DLNs were analyzed by flow cytometry. Mean value is depicted as a horizontal line among the symbols, and error bars represent SEM ( $n = 5$ ). **(A)** Representative dot plots showing CD4 (y-axis) and GM-CSF (x-axis) from control (left panel) and IVIg group (right panel). Number in each quadrant represents the percent among CD4 population. **(B)** Plot showing GM-CSF<sup>+</sup> among CD4 in DLN from control (open circles) and IVIg-treated (filled circles) mice. **(C)** Absolute number of CD4<sup>+</sup>GM-CSF<sup>+</sup> in the CNS. **(D)** Representative dot plot showing podoplanin<sup>+</sup> gated for CD4<sup>+</sup> in control (left panel) and IVIg (right panel) mice. **(E)** Percent of CD4 cells that are podoplanin<sup>+</sup> (Pdp) in DLN. **(F)** Absolute cell count of CD4<sup>+</sup>GM-CSF<sup>+</sup> in the CNS. \*\* $p < 0.01$  determined by Mann–Whitney  $U$  test.

mediated regulation of T cell populations raises a possibility that F(ab')<sub>2</sub> part of IgG molecule should be equally effective as that of intact IgG. To explore this, EAE mice were treated with IVIg or equimolar concentration of F(ab')<sub>2</sub> fragments. F(ab')<sub>2</sub> also significantly delayed the onset of EAE and decreased severity of the disease as shown by mean disease score on day 11 (Fig. 4A). Mice were sacrificed on day 9 and analyzed for CD4 T cell populations by flow cytometry. F(ab')<sub>2</sub> inhibited Th17 and Th1 cells (Fig. 4B, 4C) comparable to IVIg. MOG<sub>35–55</sub>-specific cytokine secretion assay revealed decreased amounts of IL-17 and IFN- $\gamma$  from F(ab')<sub>2</sub> or IVIg-treated mice (Fig. 4D, 4E). F(ab')<sub>2</sub> also enhanced the number of Treg in spleen (Fig. 4F) and de-

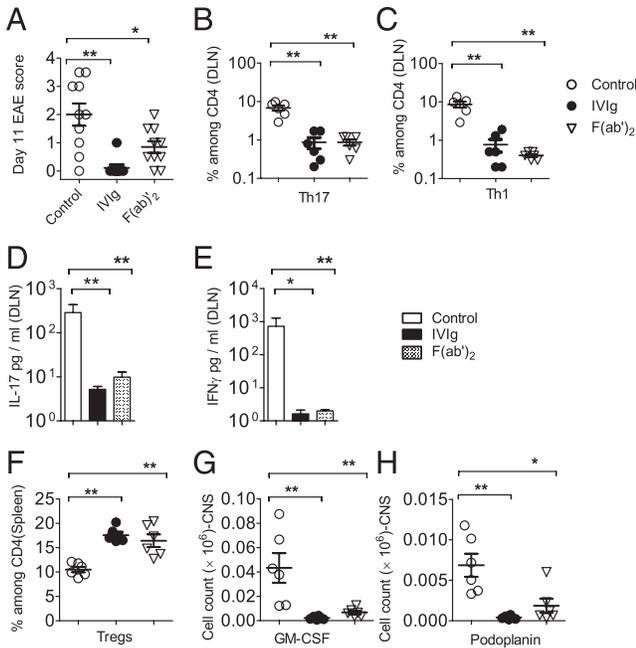


**FIGURE 3.** Inhibitory FcR Fc $\gamma$ RIIB is not required for IVIg-mediated reciprocal modulation of effector and regulatory CD4 subsets in vivo. Female Fc $\gamma$ RIIB<sup>-/-</sup> mice under C57BL/6J background were immunized with 200  $\mu$ g of MOG<sub>35–55</sub> peptide in CFA, and this was followed by pertussis toxin injection as mentioned in experimental procedures. Control mice received 0.2 M glycine (open squares); treatment group received IVIg (filled squares). Nine days after immunization, mice were sacrificed, and cells from DLN, spleen, and CNS were evaluated by flow cytometry. Mean values are depicted as a horizontal lines among the symbols, and error bars represent SEM ( $n = 4$ ). **(A)** Plots show frequency of Th17 and Th1 and Treg in DLN. **(B)** Th17 and Th1 and Treg in spleen are shown. **(C)** CD4 cells positive for GM-CSF and podoplanin in the DLN are represented. **(D)** CD4<sup>+</sup>GM-CSF<sup>+</sup> and CD4<sup>+</sup>podoplanin<sup>+</sup> in CNS at day 9 in control and IVIg groups are represented. \* $p < 0.05$  determined by Mann–Whitney  $U$  test.

creased the expression of GM-CSF and podoplanin in CD4 cells similar to IVIg (Fig. 4G, 4H), rendering them less pathogenic. These results demonstrate that in vivo reciprocal modulation of CD4 subsets by IVIg is F(ab')<sub>2</sub> dependent. These results, in addition to the above-mentioned dispensable nature of inhibitory Fc $\gamma$ RIIB for the modulatory effect of IVIg in EAE, precluded us from examining the direct role of Fc fragments.

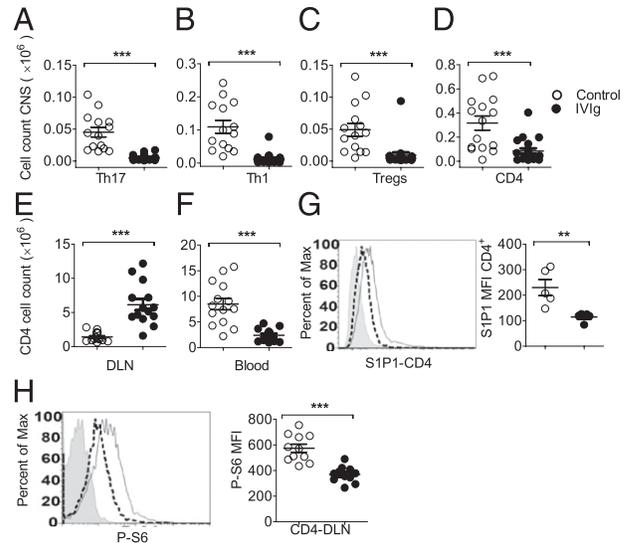
#### IVIg decreases infiltration of lymphocytes to the CNS by inhibiting their egress from the DLN

Protective effect of IVIg treatment in EAE is associated with decreased number of lymphocytes and absence of inflammatory foci in the CNS (13, 15). It has been previously shown that IVIg interferes with leukocyte recruitment to the CNS in an  $\alpha 4$  integrin-dependent manner (24). However, Th17 and Th1 cells use different strategies to invade the CNS (25, 26). To investigate the effect of IVIg on trafficking of Th17 and Th1 in EAE, brain and



**FIGURE 4.** Fragment of Ag binding F(ab')<sub>2</sub> part of IVIg also retained capacity to reciprocally modulate effector and regulatory CD4 subsets in vivo. Ten- to 12-wk-old female WT C57BL/6J mice were immunized with 200 μg of MOG<sub>35-55</sub> peptide in CFA followed by pertussis toxin as mentioned in experimental procedures. Control mice received 0.2 M glycine (open circles), IVIg group received 0.8 g/kg body weight Gamunex (filled circles) and mice in F(ab')<sub>2</sub> group received 0.5 g/kg pepsin-treated IgG-free fragment of Ag binding F(ab')<sub>2</sub> (open triangles). Mice were sacrificed on day 9, and various cell types were evaluated by flow cytometry. Mean values are depicted as a horizontal lines among the symbols and error bars represent SEM. **(A)** Mean EAE disease scores on day 11 are represented (*n* > 9). **(B)** Representation of percent of Th17 among CD4 population in DLN. **(C)** Data on Th1 DLN are shown. MOG<sub>35-55</sub>-specific cytokine secretion assay was performed by incubating cells from DLN with 10 μg/ml of MOG<sub>35-55</sub> peptide for 24 h. Supernatants were analyzed for cytokines IL-17A **(D)** and IFN-γ **(E)**. Logarithmic scales are used to show the quantity of cytokine. **(F)** Foxp3<sup>+</sup> regulatory cells among CD4<sup>+</sup> population in spleen are plotted. **(G)** CNS-infiltrating CD4<sup>+</sup>GM-CSF<sup>+</sup> cells were analyzed on the day of onset, cell counts were estimated by flow cytometry data, and absolute numbers of the same are plotted. **(H)** Representation of absolute number of CD4<sup>+</sup> podoplanin<sup>+</sup> cells in the CNS. \**p* < 0.05, \*\**p* < 0.01, determined by Mann–Whitney *U* test.

spinal cords were analyzed on the day of onset (day 9). IVIg inhibited infiltration of Th1, Th17, and Treg into the CNS (Fig. 5A–C). MOG<sub>35-55</sub>-specific cytokine secretion assay revealed decreased amounts of IL-17 and IFN-γ from the CNS of IVIg-treated mice (Supplemental Fig. 2). Accordingly, there was an overall decrease in absolute number of CD4 cells found in the CNS (Fig. 5D). Surprisingly, DLN of IVIg-treated EAE mice were bigger in size and contained 2- to 5-fold more numbers of CD4 cells than the control EAE mice (Fig. 5E). We also observed a decrease in total number of CD4 cells circulating in the blood (Fig. 5F). These data suggested that T cell entry into DLN of IVIg-treated EAE mice was intact; however, their exit from DLN was affected. To further probe into the molecular mechanisms involved in sequestering of CD4 cells into the DLN, we investigated the S1P–S1P1 axis, which controls the trafficking and migration of lymphocytes (27). IVIg treatment in EAE mice for 6 d decreased S1P1 on CD4 cells in DLN (Fig. 5G). Akt–mTOR kinase is a critical downstream pathway of S1P1 signaling. To investigate whether IVIg-mediated inhibition of the S1P–S1P1 pathway involves the mTOR pathway, we analyzed the activity of



**FIGURE 5.** IVIg decreases infiltration of CD4 lymphocytes to the CNS by inhibiting their egress from DLN involving S1P1–mTOR axis. Control and IVIg-treated EAE mice were sacrificed on day 9. To analyze the total number of circulating cells in the blood, mice were infused with PBS/EDTA, and cells were collected. Cells from Blood, DLN, spleen and CNS were analyzed by flow cytometry. Absolute numbers of each type of cells in various organs were estimated by extrapolating data from flow cytometry. Open circles represent control mice and filled circles represent IVIg-treated mice. Mean value is depicted as a horizontal line among the symbols and error bars represent SEM. \*\**p* < 0.01, \*\*\**p* < 0.001 determined by Mann–Whitney *U* test. Plots show total number of Th17 **(A)**, Th1 **(B)**, and Treg **(C)** in CNS (*n* > 10). Absolute number of CD4 cells in CNS **(D)**, DLN **(E)**, and blood **(F)** are plotted (*n* > 10). **(G)** Representative histogram (left panel) showing S1P1 on CD4 cells from DLN of control (continuous thin line) and IVIg (dashed thick line) group; autofluorescence is shown as shaded gray area. Mean fluorescence intensity (MFI) of S1P1 in CD4 cells are shown (right panel); *n* = 5. **(H)** Representative histogram (left panel) showing MFI of phospho-S6 ribosomal protein (Ser<sup>240/244</sup>) in CD4 cells from DLN of control (continuous thin line) and IVIg (dashed thick line) group; autofluorescence is shown as shaded gray area. MFI of P-S6 in CD4 cells from 10 mice are shown (right panel).

mTOR in CD4 cells from DLN by estimating the level of phospho-S6 ribosomal protein (Ser<sup>240/244</sup>) (28). CD4 cells from IVIg-treated mice displayed reduced phosphorylation of S-6 ribosomal protein as compared with the control (Fig. 5H). Additionally F(ab')<sub>2</sub> fragments also inhibit the lymphocyte egress from DLN comparable to IVIg (Supplemental Fig. 3). S1P1 is also expressed on astrocytes in the CNS and contributes to demyelination during EAE (29, 30). We checked if IVIg modulated any S1P1 on astrocytes and observed no significant change in the surface expression on astrocytes identified as FSC-A<sup>low</sup>SSC-A<sup>hi</sup> GLAST-1<sup>+</sup>CD11b<sup>-</sup> cells (Supplemental Fig. 4A, 4B). These results show that IVIg downregulates S1P1–mTOR signaling on CD4 T cells, leading to the inhibition of their egress from DLN, thus explaining the increase in the size of the DLN and decrease in infiltration of lymphocytes to CNS.

**Discussion**

Although IVIg is beneficial in the treatment of several of T cell-mediated autoimmune and inflammatory complications (31), the precise mechanisms governing the Th cell polarization and the balance between Treg and Th17 cells by IVIg have not been identified. We believe that IVIg can influence T cell functions by several multipronged mechanisms by inhibiting the differentiation and proliferation of pathogenic Th1 and Th17 cells, enhancing the expansion of Tregs and their functions, or altering the cytokine

milieu in the microenvironment or inducing apoptosis. These different functions may be mediated either by a direct interaction of IVIg with T cells or via modulating the APCs including macrophages, dendritic cells, or even B cells.

We demonstrate that administration of IVIg to EAE mice significantly reduces the severity of disease by robustly inhibiting the differentiation of Th17 and Th1 cells in both DLN and spleen (all of the mice in IVIg group showed a 4- to 5-fold decrease in Th17 and Th1 cells). Further, *ex vivo* stimulation of the lymphocytes with MOG<sub>35-55</sub> showed decreased secretion of IL-17 and IFN- $\gamma$ . We also observed a simultaneous expansion of Treg population in the spleen but not in the DLN. Our results are in line with the previous observations on the effect of IVIg on Treg and human Th17 cells *in vitro* (15, 32). Hence, the protective effect of IVIg in EAE underscores the reciprocal modulation of effector CD4 cells (Th1 and Th17) and Treg. In addition, IVIg decreases the expression of GM-CSF and podoplanin in CD4 T cells. GM-CSF produced by Th17 cells potentiates neuroinflammation by attracting myeloid cells to the CNS and upregulating their surface MHC class II (2). Podoplanin expressed on Th17 cells is involved in the formation of deleterious ectopic lymphoid structures in CNS (21, 33). By reducing the expression of these molecules in CD4 T cells, IVIg may thus inhibit the formation of detrimental inflammatory foci in CNS. The relevance of these findings in other autoimmune conditions and in patients treated with IVIg needs to be further examined.

Upregulation of inhibitory Fc $\gamma$ RIIB is implicated in the beneficial effect of IVIg (34, 35). More recently, it is shown that  $\alpha$ 2,6 sialylation on the Fc portion of IgG in IVIg upregulates inhibitory Fc $\gamma$ RIIB on macrophages through a Th2 pathway in a K/BxN serum-transfer arthritis model. Using EAE, a T cell-mediated autoimmunity, we show that IVIg is able to inhibit Th17 and Th1 cells independent of Fc $\gamma$ RIIB. Additionally, increase of Treg, inhibition of GM-CSF and podoplanin, and modulation of CD4 T cell trafficking by IVIg does not require Fc $\gamma$ RIIB. We also report that F(ab')<sub>2</sub> fragments of IVIg retained the capacity to delay the onset of EAE and reciprocally regulate CD4 subsets similar to intact IVIg. However, exploring the role of SIGNR1 (a receptor for sialylated IgG) and  $\alpha$ -2,6 sialylation on the Fc portion of IgG in this regulatory shift of CD4 subsets by IVIg is an interesting perspective.

Further, IVIg or F(ab')<sub>2</sub> fragments effectively inhibited the infiltration of Th1, Th17, and Treg to CNS, which is in consensus with previous reports (13, 15). We observed an increased accumulation of CD4 cells in the DLN by IVIg and their concomitant decrease in circulation and the target organ. We provide mechanistic evidence for this interference of lymphocyte trafficking. Differentiated T cells exit from the DLN by using a gradient of S1P across the lymphoid tissue, lymph, and blood through S1P1 (27). By downregulating S1P1 on CD4 cells, IVIg inhibits the lymphocyte egress, leading to sequestration of these cells in the DLN. This observation is similar to the effect of FTY720, a known modulator of S1P–S1P signaling, which also restricts lymphocytes in the lymph nodes (36). S1P1 acts through downstream kinases like Akt and mTOR (37). Interestingly, S1P enhances Th17 differentiation *in vitro* (38), and the S1P–mTOR axis is involved in restraining the development and function of Foxp3<sup>+</sup> T cells and promoting Th1 cells (39). We reasoned that reciprocal modulation of T cell responses by IVIg may involve the S1P–mTOR signaling pathway. Decreased activity of mTOR kinase in the CD4 cells further substantiates our hypothesis. Thus, by downregulating the S1P1–mTOR axis, IVIg may inhibit the differentiation of effector cells (Th17 and Th1) while favoring the Treg.

We have explored the possible mechanisms by which IVIg decreases S1P1. As IVIg is a polyclonal and polyspecific Ig

preparation and is known to contain Abs against a wide range of biomolecules (40) including lipids (41), we hypothesized that IVIg interacts directly with S1P in circulation to quench it, thereby disrupting the gradient of S1P across lymph node, lymph, and blood. We tested this indirectly by estimating S1P1 expression on the surface of circulating CD4 T lymphocytes during IVIg therapy. Circulating lymphocytes express the least amount of S1P1 on their surface, as they are bathed in high concentration of S1P (27). Any decrease in the concentration of S1P in the microenvironment of CD4 T cells leads to upregulation of S1P1 on their surface. We observed no significant differences in the surface expression of S1P1 on CD4 T cells in blood upon IVIg treatment during EAE (Supplemental Fig. 4C, 4D), suggesting that IVIG does not modify the level of S1P in the circulation. Hence, IVIG-mediated decrease of the expression of S1P1 and the sequestration of lymphocytes into DLN is independent of the quenching of S1P in circulation. However, the mechanisms of inhibition of T cell trafficking by IVIg can be further studied using S1P1-transgenic mice. Whether Treg-itopes have any role in reciprocal regulation of effector and Treg remains to be explored (42).

Pathogenesis of EAE involves activation and differentiation of naive neuroantigen-specific CD4 cells into Th17 and Th1 cells in the secondary lymphoid organs. These pathogenic T cells migrate to the CNS and potentiate axonal destruction by facilitating infiltration of other myeloid effector cells through GM-CSF and formation of ectopic lymphoid structures mediated by podoplanin. Our results show that IVIg-mediated inhibition of EAE implicates multiple targets acting at different phases of immune response. Thus, IVIg can inhibit the initiation of pathogenic immune response by inhibiting the polarization of naive T cells into Th17 and Th1 cells. This process is associated with a concomitant expansion of Treg *in vivo*. IVIg decreases the tissue damaging potential of pathogenic T cells by downregulating key molecules such as GM-CSF and podoplanin. Further, IVIg can circumvent neuronal degeneration by inhibiting the infiltration of CD4 T lymphocytes to the target organ by restraining their exit from the DLN through the S1P–S1P1–mTOR pathway, which may be the key target for reciprocal regulation of CD4 subsets. Together, our results provide a cellular and molecular basis underlying the beneficial effect of IVIg in certain T cell-dependent autoimmune and inflammatory conditions.

## Acknowledgments

We thank F. Canale, M. Muñoz, and S. Delignat for technical assistance.

## Disclosures

The authors have no financial conflicts of interest.

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# Inhibitory Effect of IVIG on IL-17 Production by Th17 Cells is Independent of Anti-IL-17 Antibodies in the Immunoglobulin Preparations

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Received: 4 July 2012 / Accepted: 24 July 2012 / Published online: 7 August 2012  
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## Abstract

**Purpose** Th17 cells and their cytokines play a critical role in the pathogenesis of various autoimmune and inflammatory diseases. Recently, we have demonstrated that intravenous immunoglobulin (IVIG) suppresses differentiation, amplification, and functions of human Th17 cells. In this report we investigated whether IVIG inhibits IL-17 production by Th17 cells cultured in the presence of IL-23 and whether the inhibitory effect of IVIG on IL-17 production implicates anti-IL-17 antibodies.

**Methods** Naive CD4<sup>+</sup> T cells were stimulated in the presence of TGF-β, IL-21, and IL-23 for the differentiation of Th17 cells. Memory CD4<sup>+</sup> T cells were stimulated with IL-1β, IL-6, and IL-23 for the amplification of Th17 cells. IVIG (0.15 mM) was added to the cells 12 h after initiation of cultures. IL-17A cytokine and anti-IL-17 antibodies were measured by ELISA.

**Results** IL-23 did not deter the inhibitory effect of IVIG on IL-17 production from the differentiating and expanding Th17 cells. Further, suppression of IL-17 by IVIG did not

implicate anti-IL-17 antibodies in the immunoglobulin preparations.

**Conclusion** The effect of IVIG on the inhibition of IL-17 production by Th17 cells is a consequence of modulation of Th17 cells and their intracellular signaling pathways and not due to passive neutralization of IL-17 by anti-IL-17 antibodies in the immunoglobulin preparations.

**Keywords** Th17 · IL-17 · IVIG · anti-IL-17 antibodies · IL-23

## Introduction

CD4<sup>+</sup> T cells are effector cells of the immune system that coordinate the complete array of immune responses. In addition to regulating the immune responses and conferring protection against invading pathogens, CD4<sup>+</sup> T cells can also mediate inflammatory and autoimmune diseases under certain conditions. CD4<sup>+</sup> T cells are heterogeneous and

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show plasticity. In addition to natural CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells (nTregs), naive CD4<sup>+</sup> T cells can be differentiated into Th1, Th2, and induced Tregs (iTregs) in the periphery under the influence of antigen-presenting cells (APCs) and appropriate cytokine milieu. Recently, Th17 cells—a distinct lineage of CD4<sup>+</sup> T cells that express effector molecule IL-17 and transcription factor RORC (or ROR $\gamma$ t in mice)—have been identified [1].

Th17 cells require STAT3 signaling for differentiation. They secrete effector molecules IL-17A, IL-17F, GM-CSF, IL-21, IL-22, and CCL20. Th17 cells and their effector molecules can act on both immune and non-immune cells and exert a wide range of functions, including activation and proliferation of cells, stimulation of the secretion of inflammatory mediators, immunoglobulin class switching in B cells, cytotoxic T cell functioning, secretion of antimicrobial peptides by epithelial cells, and recruitment of inflammatory cells [1]. Th17 cells are critical for protection against extracellular microbes and fungi. However, when their functions are dysregulated, Th17 cells can also mediate autoimmune and inflammatory diseases. In fact, Th17 cells and their cytokines have been implicated in the pathogenesis of several diseases, including multiple sclerosis, systemic lupus erythematosus, Kawasaki disease, vasculitis, dermatomyositis, asthma, and rheumatoid arthritis [1, 2].

Intravenous immunoglobulin (IVIG) is a therapeutic preparation of normal IgG obtained from the plasma pools of a large number of healthy donors [3]. In addition to replacement therapy of primary immunodeficiencies, IVIG also is used in the therapy of many autoimmune diseases, including immune thrombocytopenic purpura, Kawasaki disease, pemphigus, chronic inflammatory demyelinating polyneuropathy, dermatomyositis, vasculitis, and myasthenia gravis [3, 4]. IVIG can exert therapeutic benefits in these diverse diseases via numerous mutually nonexclusive mechanisms [3, 5–9]

Recently, we have demonstrated that IVIG suppresses the differentiation and amplification of Th17 cells, as well as the secretion of their effector molecules IL-17, IL-21, IL-22, and CCL20 [10, 11]. These effects of IVIG are associated with inhibition of RORC, phosphorylation of STAT3, and reciprocal regulation of Tregs. Since Th17 cells are implicated in the pathogenesis of various diseases where IVIG is also beneficial, our results indicate that inhibition of Th17 cells represents one of the major mechanisms of action of IVIG.

It is known that IVIG contains specific, high-avidity antibodies to several cytokines [3]. Therefore it is likely that observed inhibition of IL-17 production by IVIG might be due to passive neutralization of this cytokine by anti-IL-17 antibodies in the immunoglobulin preparations. In this report we aimed at identifying whether the effect of IVIG on the inhibition of IL-17 production by Th17 cells is a consequence of modulation of Th17 cells and their intracellular signaling or due to passive neutralization.

## Methods

### Cell-Culture Reagents and Antibodies

The monoclonal antibodies (mAbs) anti-CD3 (clone UCHT1) and anti-CD28 (clone 37407), and the cytokines TGF- $\beta$ , IL-1 $\beta$ , IL-23, and IL-6 were purchased from R&D systems (Lille, France). IL-21 was from ImmunoTools (Friesoythe, Germany).

### T Cell Purification

Peripheral blood mononuclear cells were obtained from buffy bags of healthy donors as per the standard protocol of density gradient centrifugation using LSM 1077 (PAA Laboratories, Les Mureaux, France). Untouched total CD4<sup>+</sup> T cells were isolated by negative selection using the CD4<sup>+</sup> T cell isolation kit II (Miltenyi Biotech, Paris, France). Next, naive (CD45RO<sup>-</sup>) and memory (CD45RO<sup>+</sup>) CD4<sup>+</sup> T cells were separated using CD45RO microbeads (Miltenyi Biotech). CD4<sup>+</sup>CD25<sup>-</sup>CD45RO<sup>-</sup> naive T cells were then obtained by depletion of CD25<sup>+</sup> cells using CD25 microbeads (Miltenyi Biotech). The purity of the various cell populations was >95 %.

### Th17 Cell Differentiation and Amplification Using Cytokines in Combination with IL-23

Ninety-six well U-bottom plates were coated with 1.5  $\mu$ g/ml of anti-CD3 mAb by incubating for 16 to 18 h at 4 °C. Antibody solution was then removed and naive or memory CD4<sup>+</sup> T cells ( $5 \times 10^4$  per well/200  $\mu$ L) in serum-free X-VIVO 15 medium were added to the wells. The cells were stimulated with 1.0  $\mu$ g/mL soluble anti-CD28 mAb in the presence of cytokines for the differentiation and expansion of Th17 cells. The cytokines for the differentiation of Th17 cells from naive T cells included TGF- $\beta$  (5 ng/mL), IL-21 (25 ng/mL), and IL-23 (25 ng/mL). For the expansion of Th17 cells within memory T cells, a combination of IL-1 $\beta$  (12.5 ng/mL), IL-6 (25 ng/mL), and IL-23 (25 ng/mL) was used. IVIG (0.15 mM) was added to the cells after 12 h post initiation of cultures. The stimulated T cells were cultured for 5 to 6 days. Supernatants were collected at the end of culture for the analysis of secreted IL-17A.

### Measurement of IL-17A

IL-17A in the cell-free culture supernatants or in IVIG preparations was quantified by DuoSet ELISA kit (R&D Systems).

### Detection of Anti-IL-17 Antibodies in IVIG

We used IL-17 DuoSet ELISA with suitable modifications for identifying anti-IL-17 antibodies in the IVIG

preparations. To the capture antibody (mouse anti-human IL-17 monoclonal antibodies)-coated microplate wells, either IL-17 standards or 1:1 mixture of 1000 pg/mL IL-17 and assay diluent, or 1:1 mixture of 1000 pg/mL IL-17 and IVIG preparations (IgG concentration 100 mg/mL) were added. Thus, the final concentration of IL-17 in each well was 500 pg/mL. If IVIG preparations contain antibodies to IL-17, then these antibodies compete with coated capture antibodies for binding to IL-17; therefore, the quantity of IL-17 detected will be decreased. After 2 hours of incubation, microplates were washed, and biotinylated goat anti-human IL-17 antibodies were added. The plates were again incubated for 2 h, washed, and streptavidin-HRP was added. The enzyme activity was revealed by adding substrate solution, and reactions were measured by microplate reader.

#### Statistical Analysis

Paired Student's *t*-test was used to determine the statistical significance of the data.  $P < 0.05$  was considered significant.

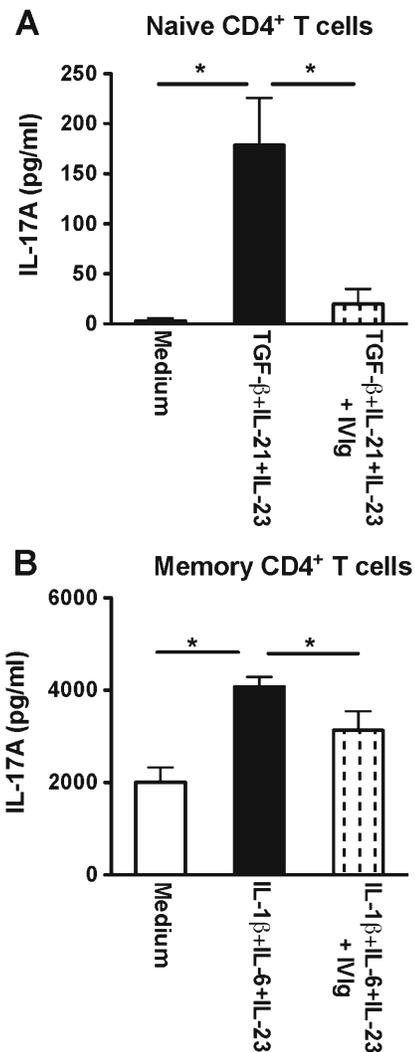
#### Results

##### IL-23 Does not Deter the Inhibitory Effect of IVIG on IL-17 Production from Differentiating and Expanding Th17 Cells

Recently we have demonstrated that IVIG inhibits IL-17 production from Th17 cells that have been differentiated in the presence of TGF- $\beta$  and IL-21 or expanded with IL-1 $\beta$  and IL-6 [10, 11]. However, these culture conditions did not include IL-23, an inflammatory cytokine critical for the survival and stabilization of differentiated Th17 cells [1]. Therefore, we explored whether IVIG exerts a suppressive effect on Th17 cells even in the presence of IL-23.

IVIG was added at 0.15 mM concentration to the T cells 12 h after initiation of cultures. To simulate an *in vivo* situation, we did not add neutralizing antibodies to IFN- $\gamma$  and IL-4 to Th17 cultures, the cytokines that are known to inhibit the programming of Th17 cells. After 5- to 6-day cultures, the culture supernatants were analyzed for the quantity of IL-17 secreted.

As compared with the naive CD4<sup>+</sup> T cells cultured with polarizing cytokines, cells stimulated with CD3 and CD28 antibodies alone did not produce significant amounts of IL-17. This indicates that, in the absence of polarizing cytokines, naive T cells do not differentiate into Th17 cells by default (Fig. 1a). However, CD3 and CD28 stimulation was sufficient to activate memory Th17 cells to produce significant quantities of IL-17 (Fig. 1b), the production of which was enhanced two-fold when IL-1 $\beta$ , IL-6, and IL-23 were added to the cultures. As expected, expanding memory



**Fig. 1** IL-23 does not deter the inhibitory effect of IVIG on IL-17 production from differentiating and expanding Th17 cells. CD4<sup>+</sup> T cells were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of cytokines. IVIG (0.15 mM) was added 12 hours following initiation of culture, and supernatant was collected to determine the level of IL-17A secretion. **a** Naive CD4<sup>+</sup> T cells were stimulated in the presence of TGF- $\beta$ , IL-21, and IL-23 for the differentiation of Th17 cells. **b** Memory CD4<sup>+</sup> T cells were stimulated with IL-1 $\beta$ , IL-6, and IL-23 for the amplification of Th17 cells. Data from 5 donors is presented (mean  $\pm$  SEM). \*Statistical significance as determined by paired *t*-test, where  $P < 0.05$

Th17 cells produced several-fold higher amounts of IL-17 compared with differentiating Th17 cells.

Furthermore, IVIG significantly suppressed the amount of IL-17 secreted by differentiated Th17 cells (80 % reduction compared with cytokine control), while a 40 % decline in the production of IL-17 was observed in expanding Th17 cells (Fig. 1a and b). The suppressive effect of IVIG was more pronounced on differentiating Th17 cells. These results together indicate that IL-23 does not deter the

inhibitory effect of IVIG on IL-17 production from differentiating and expanding Th17 cells.

#### Inhibitory Effect of IVIG on IL-17 Production by Th17 Cells Does not Implicate Anti-IL-17 Antibodies in the Immunoglobulin Preparations

Our previous results demonstrate that IVIG inhibits the percentage of cells positive for IL-17A and RORC (as analyzed by flow cytometry) [10], thus indicating that IVIG subdues IL-17 production by modulating intracellular signaling, including inhibition of STAT3 phosphorylation. However, IVIG is known to contain antibodies to various self-motifs, including cell surface molecules and cytokines [3]. These anti-cytokine antibodies can inhibit the activity of specific cytokines. Therefore, it is likely that reduced quantities of IL-17 in the cell-free culture supernatants of IVIG-treated Th17 cells might reflect the passive neutralization/blockade of IL-17 by anti-IL17 antibodies in the immunoglobulin preparations. Therefore, we investigated the presence of anti-IL-17 antibodies in the IVIG preparations.

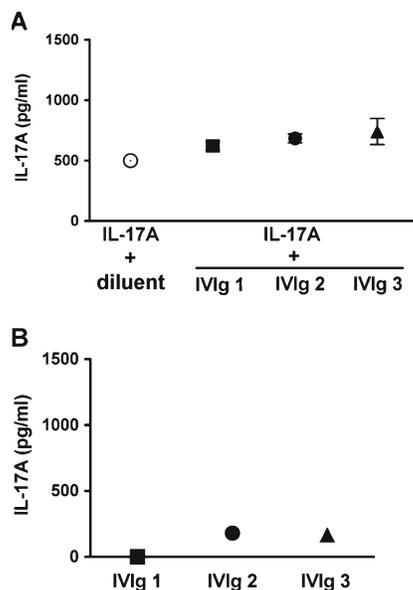
As shown in Fig. 2a, when 3 different IVIG preparations were individually mixed with a known quantity of IL-17A, there was no reduction in the quantity of IL-17A detected. These results indicate that IVIG preparations do not contain inhibitory antibodies to IL-17A, thereby suggesting that the suppression of IL-17 production by IVIG was due to

modulation of Th17 cells rather than to passive neutralization of secreted IL-17. Interestingly, slightly increased amounts of IL-17A were detected when IVIG preparations were mixed with a known amount of IL-17A. Therefore, we analyzed IL-17A in the IVIG preparations. We found that 2 (out of 3) IVIG preparations contain minute amounts of IL-17A (Fig. 2b).

#### Discussion and Conclusions

It is established that TGF- $\beta$  holds the central role in the differentiation of both Th17 and iTregs. Thus, TGF- $\beta$  induces both RORC, a lineage-specific transcription factor for Th17 cells, and FoxP3, a lineage-specific transcription factor for Tregs. However, in the absence of inflammation and in the presence of high concentrations of TGF- $\beta$ , FoxP3 inhibits RORC—leading to the differentiation of naive T cells toward iTregs. In contrast, under inflammation, stimulation of cells by IL-21 ignites phosphorylation of STAT3 [1]. Phosphorylated STAT3 releases RORC from FoxP3 and enhances the expression of RORC. Further, both RORC and phosphorylated STAT3 cooperate to induce effector cytokines of Th17 cells. IL-21 acts in an autocrine manner to amplify the differentiation process of Th17 cells. In addition, TGF- $\beta$  plus IL-6/IL-21 signaling leads to the expression of IL-23 and IL-1 $\beta$  receptors on differentiating Th17 cells. IL-23, IL-1 $\beta$ , and IL-6 stabilize the Th17 cell population and help to acquire effector functions. Thus, IL-23 is one of the key cytokines that play a critical role in the survival, stabilization, and acquisition of effector functions of Th17 cells [1].

Our results demonstrate that IVIG inhibits Th17 cell differentiation and expansion even in the presence of stabilizing factor IL-23, and may explain in part the beneficial effects of IVIG therapy in those autoimmune and inflammatory diseases in which Th17 cells and IL-17 play a central role in pathogenesis. In fact, our preliminary results also indicate that IVIG therapy is associated with a reduced number of Th17 cells in the peripheral blood and decreased amounts of IL-17A in the sera of autoimmune patients such as those with Guillain–Barré syndrome and dermatomyositis. Furthermore, our results show that this inhibitory effect of IVIG on IL-17 production involves direct modulation of T cell functions [10, 11] and does not implicate anti-IL-17 antibodies. However, our assay has inherent limitation and the only anti-IL-17A antibodies that would be detected in the IVIG preparations would be those cross-react with the epitope recognized by the mouse anti-human IL-17 monoclonal capture antibodies or numerous epitopes that are detected by the goat anti-human IL-17 polyclonal detector antibodies. Therefore, we do not absolutely rule out the presence of anti-IL-17A antibodies in three different IVIG preparations that are tested in this report.



**Fig. 2** IVIG does not contain anti-IL-17 antibodies. **a** 1:1 mixture of 1000 pg/mL IL-17A plus 100  $\mu$ L assay diluent or 100  $\mu$ L IVIG preparations (IgG concentration 100 mg/mL) were added to the mouse anti-human IL-17 (capture antibody)-coated microplate wells. The quantity of IL-17A bound to coated capture antibodies was determined by ELISA. **b** Amount of IL-17A (pg/mL) in the IVIG preparations (IgG concentration 100 mg/mL)

On the contrary, we found that 2 IVIG preparations contain minute amounts of IL-17. Interestingly, IL-17-neutralizing antibodies are associated with predisposition towards fungal infections [12, 13]. The lack of anti-IL-17 antibodies in IVIG may explain the fact that IVIG therapy does not predispose autoimmune and primary immunodeficient patients to fungal infections.

IVIG can inhibit Th17 cells both directly and indirectly. The direct effects of IVIG are due to the interaction of IgG molecules with Th17 cells. Thus, IVIG inhibits production of effector molecules of Th17 cells such as IL-17A, IL-17F, IL-22, and CCL20; STAT-3 phosphorylation; and RORC expression [10, 11]. However, IVIG can also affect Th17 cell functions indirectly by modulating other immune cells. CD4<sup>+</sup> T cells in general are dependent on APCs for their polarization. Thus, interaction of TCR complex with HLA-DR (Signal 1); CD28 with CD80/CD86, CD2 with CD58, and CD11a/CD18 with CD54 (Signal 2); and polarizing cytokines produced by activated APCs and other immune cells in the local environment determine the polarization of CD4<sup>+</sup> T cells [14]. Several reports have demonstrated that IVIG inhibits the maturation (HLA-DR and co-stimulatory molecules) and activation (secretion of inflammatory and polarizing cytokines) of APCs such as dendritic cells [15, 16]. Thus, suppression of all three signals on APCs required for CD4<sup>+</sup> T cell polarization can inhibit Th17 differentiation and expansion. Alternatively, the Th17 cell inhibition by IVIG might implicate expansion of Tregs with potent suppressive functions [17–22].

**Acknowledgments** Supported by grants from Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie, Université Paris Descartes and European Community's Seventh Framework Programme [FP7-2007-2013] under Grant Agreement N° HEALTH-F2-2010-260338-ALLFUN.

**Conflict of interest** The research activities on IVIG are partly supported by research grants from Octapharma AG, CSL Behring, Laboratoires Français de Fractionnement et des Biotechnologies, and Talecris Biotherapeutics.

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# Impact of gp120 on Dendritic Cell-Derived Chemokines: Relevance for the Efficacy of gp120-Based Vaccines for HIV-1

Dendritic cells (DC) are the professional antigen-presenting cells and act as sentinels of the immune system. Therefore, the activation status of DC upon an encounter with vaccine antigens determines the intensity and duration of protective immune responses. DC are also critical for the transmission of HIV-1 to CD4<sup>+</sup> T cells (2).

gp120 mediates binding of HIV-1 to DC and is one of the major antigens implicated in the pathogenesis of AIDS (12). In about 62% of individuals, gp120 can induce interleukin 10 (IL-10) in monocyte-derived DC (Mo-DC) (13). Although gp120-mediated abnormal maturation and functional alteration in Mo-DC was reported (7), we found that gp120 did not impart any functional abnormalities to Mo-DC (14). In plasmacytoid DC, gp120 can induce indoleamine 2,3-dioxygenase (4) and can inhibit TLR-9-mediated alpha interferon (IFN- $\alpha$ ) secretion (11).

During HIV infection, immature DC capture HIV at mucosal sites of initial infection and are facilitated by gp120 (6). Therefore, gp120 has been explored as one of the vaccine candidates for HIV-1 (8, 9). However, the repercussion of interaction with gp120 on the secretion of DC-derived chemokines is not known. Chemokines mediate trafficking of diverse immune cells and,

hence, are critical for the efficacy of the vaccines and for inducing protective immune responses. Upon maturation and activation, DC secrete a wide range of chemokines that dictate the migration of T cells, monocytes/macrophages, and granulocytes and, hence, can influence the course of HIV-1 infection and immune response to vaccines. Therefore, we hypothesized that gp120 vaccination can affect the immune response by modulating the chemokine expression pattern of human DC.

Immature Mo-DC were obtained from healthy donors as previously described upon ethical committee approval (3). We first performed a dose-response analysis of recombinant gp120 protein from HIV-1 on DC maturation by using various concentra-

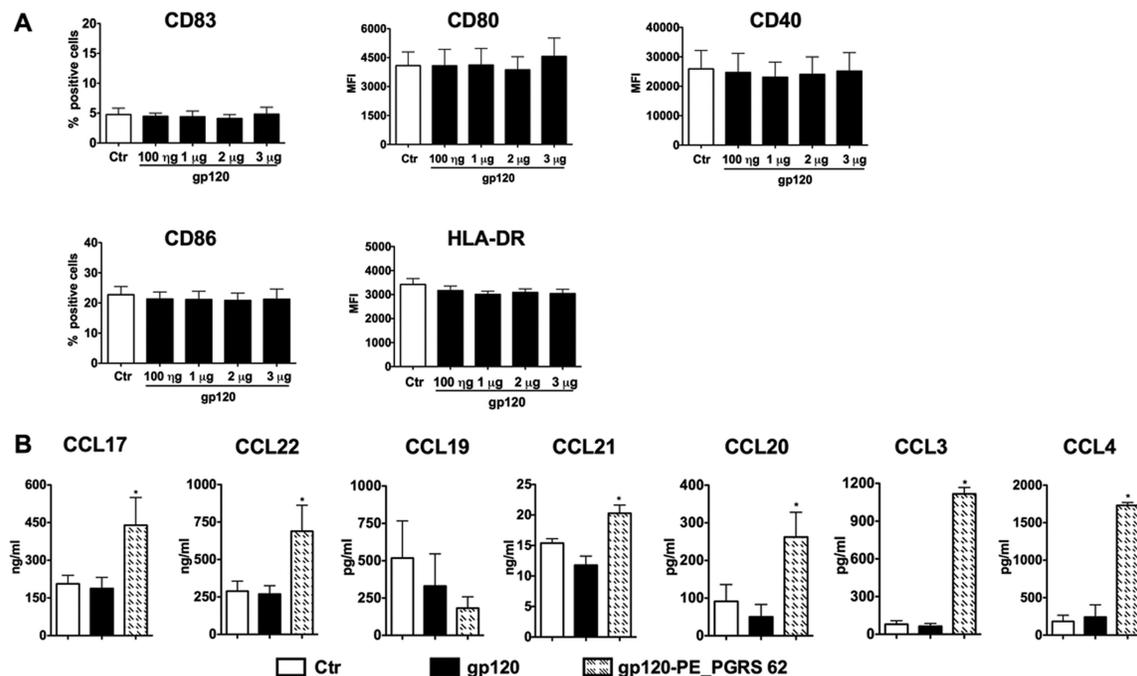
Published ahead of print 27 June 2012

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doi:10.1128/CI.00244-12



**FIG 1** Effect of gp120 from HIV-1 on human DC chemokine responses. Monocytes from buffy bags of healthy blood donors were isolated by using CD14 microbeads (Miltenyi Biotec, France). Monocytes were then differentiated into immature DC by culturing for 6 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) (1,000 IU/10<sup>6</sup> cells) and IL-4 (500 IU/10<sup>6</sup> cells). (A) Dose-response analysis of the effect of gp120 on DC maturation. Immature DC (0.5 × 10<sup>6</sup> cells/ml) were cultured in the presence of GM-CSF and IL-4 alone (Ctr) or the cytokines and 100 ng, 1 µg, 2 µg, or 3 µg of recombinant, CHO cell-expressed gp120 from HIV-1 strain JR-FL (gp120) (*n* = 3) for 48 h. The phenotype of the DC was analyzed by flow cytometry (LSR II; BD Biosciences) by using fluorochrome-conjugated monoclonal antibodies to the indicated markers. MFI, mean fluorescence intensity. (B) Immature DC (0.5 × 10<sup>6</sup> cells/ml) were cultured in the presence of GM-CSF and IL-4 alone (Ctr), the cytokines and 3 µg/ml of recombinant gp120 (gp120) (*n* = 8 to 10 donors), or a combination of the cytokines, gp120, and 5 µg/ml of the recombinant PE\_PGRS 62 protein for 48 h (gp120-PE\_PGRS 62) (*n* = 4 to 5 donors). Chemokines were quantified in the cell-free culture supernatants by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, France). We confirm that the stimulatory capacity of PE\_PGRS 62 is not due to endotoxin contamination. Statistical significance as determined by a two-tailed Student *t* test is indicated (\*, *P* < 0.05).

tions: 100 ng, 1  $\mu$ g, 2  $\mu$ g, and 3  $\mu$ g. We found that irrespective of the concentration used in the assay, gp120 did not significantly modify the maturation of DC (Fig. 1A). Therefore, for analyzing the stimulatory effect on DC-derived chemokines in subsequent experiments, we used gp120 at a concentration of 3  $\mu$ g. This concentration of gp120 is also compatible with the amount of gp120 used for immunization purposes (8–10).

Immature DC ( $0.5 \times 10^6$ ) were cultured with 3  $\mu$ g of gp120 for 48 hours, and the secretion of various chemokines was analyzed. The chemokines that were analyzed include the following: CCL22 and CCL17, the chemokines that induce migration of CCR4<sup>+</sup> cells, including Th2 cells and subsets of macrophages, DC, Th17 cells, and regulatory T cells; CCL19 and CCL21, which bind to CCR7, and CCL20, which binds to CCR6, all of which can mediate effective T cell responses to HIV-1 and vaccines; and CCL3 and CCL4, chemokines that target the recruitment of CCR5-positive immune cells, including T cells and natural killer cells, and, hence, are critical for effective immune responses toward vaccines and infectious agents (1).

We found that gp120 did not modulate any of the analyzed DC-derived chemokines. Thus, the levels of secretion of CCR5, CCR4, CCR7, and CCR6 ligands by gp120-stimulated DC were similar to that of unstimulated cells (Fig. 1B). However, gp120-exposed DC are neither defective nor tolerogenic, as stimulation of cells with a combination of gp120 and one of the immunogenic proteins, PE\_PGRS 62 (Rv3812), of *Mycobacterium tuberculosis* (5) induced significant amounts of several chemokines, including CCL3, CCL4, CCL17, CCL20, and CCL22 (Fig. 1B). Also, we observed neither synergy nor antagonism between gp120 and PE\_PGRS 62 in their capacity to stimulate DC chemokines.

Thus, a lack of enhancement of CCR5, CCR7, and CCR6 ligands in DC by gp120 indicates that gp120 does not induce migration and trafficking of inflammatory cells and T cells and, hence, does not promote effective cellular responses to HIV-1 and vaccines. As immunogenicity of vaccine antigens is one of the major criteria for inducing effective immune response to vaccines, these results, along with our previous data on the inability of gp120 to stimulate DC maturation, cytokine response, and DC-mediated T cell responses (14), might explain, in part, the possible reasons for the failure of gp120-based HIV-1 vaccines to confer protection in clinical trials (8, 9).

## ACKNOWLEDGMENTS

We thank Progenics Pharmaceuticals, Inc., and William Olson for providing recombinant gp120 of HIV-1 strain JR-FL.

This study was supported by the Centre National de la Recherche Scientifique (S.V.K.), the Institut National de la Santé et de la Recherche Médicale, Université Pierre et Marie Curie, and Université Paris Descartes (S.V.K. and J.B.), and grants from the Indo-French Center for the Promotion of Advanced Research (reference number 4803-D) (J.B.) and the Indian Institute of Science (M.S.S.).

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## B cells are resistant to immunomodulation by 'IVIg-educated' dendritic cells

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### ARTICLE INFO

Available online 16 August 2011

### ABSTRACT

Intravenous immunoglobulin (IVIg) can exert beneficial effects in autoimmune and inflammatory diseases via several mutually non-exclusive mechanisms. While, IVIg can directly modulate the functions of both innate and adaptive immune cells such as dendritic cells (DC), macrophages, B and T cells, several reports have also highlighted that the regulation of immune responses by IVIg can be indirect. In view of these results, we aimed at exploring whether indirect regulation of immune cells by 'IVIg-educated' innate cells is a universal phenomenon. We addressed this question by deciphering the modulation of B cell functions by 'IVIg-educated' DC. Our results indicate that human B cells are resistant to immunomodulation by 'IVIg-educated' DC. However, IVIg at therapeutic concentrations can directly inhibit B cell activation and proliferation. These results thus suggest that, indirect modulation of immune cells by IVIg is not a universal phenomenon.

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### 1. Introduction

Autoimmune diseases of either systemic or organ-specific nature affect substantial proportion (around 3%) of world population. These disorders cause significant morbidity, mortality and economy burden. The causes for emergence of autoimmunity can be genetic, epigenetic or environmental [1–8]. Intravenous immunoglobulin (IVIg) has been used as a one of the major immunomodulators to treat large range of autoimmune and inflammatory diseases [9–15].

IVIg exerts beneficial effects in autoimmune and inflammatory diseases via several mutually non-exclusive mechanisms [9,16–20]. Thus, IVIg can directly modulate the functions of both innate and adaptive immune cells [21–28]. Several recent reports have also highlighted that the regulation of immune responses by IVIg can be indirect [29–34]. Thus, IVIg suppresses the activation and functions of dendritic cells (DC), the sentinels of the immune system and these IVIg-modulated DC in turn inhibit the activation and proliferation of CD4+ T cells and modulate platelet functions [29,32,33]. Similarly, 2, 6-sialylated Fc-fragment of IVIg has been shown to interact with SIGN-R1 on CSF-1-dependent regulatory murine macrophages to induce Th2 cytokines via stimulation of basophils [35]. These reports thus show that IVIg modulates the functions of innate immune cells

such as DC or macrophages and these 'IVIg-educated' innate cells in turn indirectly affect other immune cell functions.

DC can regulate the immune responses through interaction with T and B lymphocytes. Thus, DC can synchronize B cell growth, activation and secretion of immunoglobulins. Since DC are professional antigen presenting cells (APC) and orchestrate immune responses via secreting wide range of cytokines and chemokines, DC also play a critical role in the pathogenesis of autoimmune diseases [36]. B cells on the other hand can regulate the maturation and functions of DC and contribute to autoimmune process by producing autoantibodies, by functioning as APC and stimulating T cell activation, and by secretion of soluble mediators.

In the present study, we aimed at exploring whether indirect regulation of immune cells by 'IVIg-educated' innate cells [29–34] is a universal phenomenon. We addressed this question by deciphering the modulation of B cell functions by 'IVIg-educated' DC. Our results indicate that IVIg at therapeutic concentrations can directly inhibit the B cell activation and proliferation. However B cells are resistant to immunomodulation by 'IVIg-educated' DC.

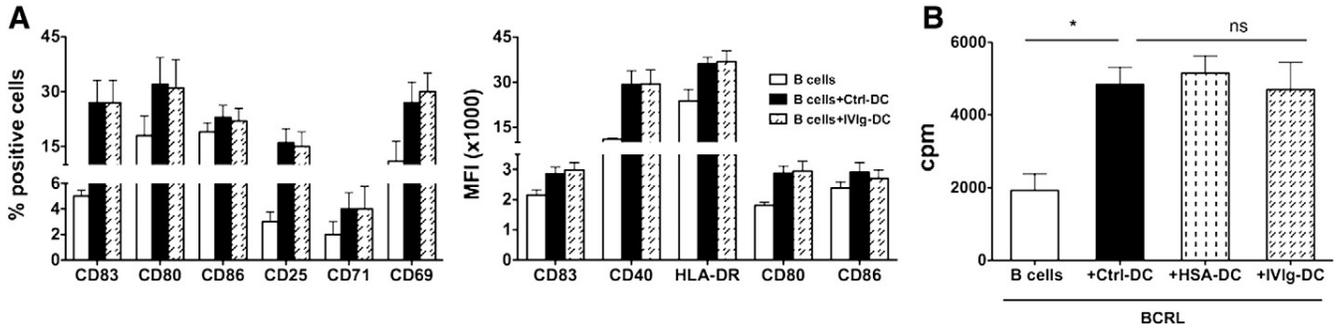
### 2. Materials and methods

#### 2.1. Generation of human DC

Monocytes purified from peripheral blood mononuclear cells of healthy individuals using CD14 microbeads (Miltenyi Biotec, France) were cultured with GM-CSF (1000 IU/million cells) and IL-4 (500 IU/million cells) to differentiate into monocyte-derived DC.

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**Fig. 1.** IVIg-educated DC do not modulate human B cell function. Monocyte-derived immature DC were cultured in the presence of GM-CSF and IL-4 alone (Ctrl-DC) or cytokines and equimolar concentration (0.15 mM) of IVIg (IVIg-DC) or HSA (HSA-DC) for 48 h. The cells were washed thoroughly after IVIg treatment. (A) The DC were co-cultured with B cells for 48 h to analyze the expression of surface markers (% positive cells and mean fluorescence intensities (MFI)) by flow cytometry. The expression level of markers on B cells induced by Ctrl-DC vs IVIg-DC is not significant. Results (mean±SEM) are from four healthy donors. (B) To assess the B cell proliferation induced by DC, BCR-stimulated B cells were cultured alone or co-cultured with DC. Cell proliferation was measured by tritiated [<sup>3</sup>H]-thymidine incorporation and presented as counts per minute (cpm). The statistical significance as determined by Student-t-test is indicated (\*, P<0.05; ns, not significant). Results (mean±SEM) are from seven healthy donors.

2.2. Effect of IVIg-treated DC on B cells

Five-day old immature DC ( $0.5 \times 10^6$  cells/ml) were cultured in the presence of GM-CSF and IL-4 alone (Ctrl-DC) or cytokines and equimolar concentration (0.15 mM) of IVIg Octagam® (Octapharma, Vienna, Austria) (IVIg-DC) or human serum albumin (HSA), a protein control for IVIg (HSA-DC) for 48 h. IVIg was dialyzed before use in experiments to remove stabilizing agents. DC were washed thoroughly after treatment and co-cultured with peripheral human blood CD19+ B cells (0.1 million cells/well/200 µl) (isolated by using CD19 microbeads, Miltenyi Biotec) at 1:5 ratio for 48 h to analyze the expression of surface markers by flow cytometry (LSR II, BD Biosciences).

To assess the B cell proliferation induced by DC, B cells 25 000 cells/well/200 µl stimulated with BCR-cross-linker (BCRL) (10 µg/ml of F(ab')<sub>2</sub> anti-human IgM, Jackson ImmunoResearch Laboratories) were co-cultured with DC at 5:1 ratio for 96 h. Cell proliferation was measured by tritiated [<sup>3</sup>H]-thymidine incorporation and presented as counts per minute (cpm).

2.3. Effect of IVIg on B cell surface markers and proliferation

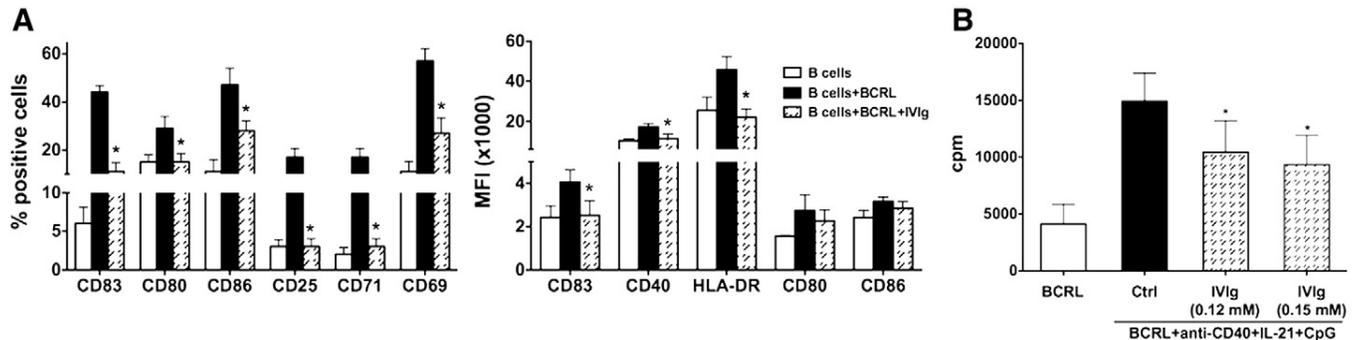
CD19+ B cells (25 000 cells/well/200 µl) were stimulated with BCRL (10 µg/ml) alone or treated with IVIg (0.15 mM) for 48 h to analyze the expression of surface markers by flow cytometry. In addition, CD19+ B cells were either stimulated with anti-CD40 MAb (5 µg/ml) + IL-21

(25 pg/ml) + CpG (2.5 µg/ml) or treated with IVIg at 0.12 and 0.15 mM concentration along with the stimulants to assess B cell proliferation.

3. Results and discussion

We found that human DC provide stimulatory signals to B cells. Thus, DC significantly enhance the activation of CD19+ B cells as analyzed by the expression of CD83, CD25 and CD69, antigen presenting molecule HLA-DR and co-stimulatory molecules CD40, CD80 and CD86 (Fig. 1A). In addition, DC also induced significant proliferation of B cells (Fig. 1B). However, DC that were pre-treated with IVIg at therapeutic concentrations, did not modulate B cell activation and proliferation (Fig. 1A and B). The expression of activation markers on B cells that were co-cultured with IVIg-treated DC was similar to those B cells co-cultured with control DC. HSA, used as a protein control for IVIg had no significant effect on DC-mediated B cell functions.

We then analyzed the direct effect of IVIg on B cell activation and proliferation. BCR-stimulation of B cells leads to significant enhancement of activation-associated molecules on B cells. Interestingly and in contrast to co-culture results with DC (Fig. 1), IVIg significantly inhibited the activation of BCR-stimulated B cells (Fig. 2A). Thus, IVIg significantly inhibited the expression of CD83, CD80, CD86, CD25, CD71, CD69, HLA-DR and CD40 on B cells. In addition, IVIg in a dose-dependent manner inhibited the proliferation of B cells mediated by combination of anti-CD40 MAb, IL-21 and CpG (Fig. 2B).



**Fig. 2.** IVIg directly affects B cell activation and proliferation. (A) CD19+ B cells were cultured alone or stimulated with BCR-cross-linker or BCR-cross-linker plus IVIg (0.15 mM) for 48 h. The expression of surface markers (% positive cells and MFI) was analyzed by flow cytometry (B) To assess the direct effect of IVIg on B cell proliferation, CD19+ B cells were either stimulated with BCR-cross-linker alone or with stimulants anti-CD40 MAb + IL-21 + CpG. In addition, IVIg was added at 0.12 and 0.15 mM concentrations along with the stimulants. The statistical significance as determined by Student-t-test is indicated (\*, P<0.05). Results (mean±SEM) are from four healthy donors.

Together, results from other laboratories [21,22,28,37–41] and the present study thus demonstrate that IVIg-mediated modulation of B cell function is due to its direct effect on B cells and not via modulation of innate immune cells such as DC. Therefore, indirect modulation of immune cells by IVIg as reported in several models [29–34] is not a universal phenomenon.

### Acknowledgments

Supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Centre National de la Recherche Scientifique (CNRS), Université Pierre et Marie Curie and Université Paris Descartes, European Community's Seventh Framework Programme [FP7/2007–2013] under Grant Agreement No: 260338 ALLFUN and Octapharma, Lachen, Switzerland.

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### The in vivo effect of Tocilizumab therapy on antibody-forming cells in patients affected with rheumatoid arthritis

Interleukin-6 (IL-6) induces in vitro differentiation of B lymphocytes into antibody-forming cells. Tocilizumab is an anti-IL-6 receptor inhibitor and was recently licensed for the treatment of rheumatoid arthritis (RA). However there is actually no data available concerning the influence of IL-6 inhibition on the B cell compartment *in vivo*. Roll P, et al. (*Arthritis Rheum* 2011; 105:1255–64) prospectively evaluated 16 RA patients who underwent Tocilizumab therapy (4–8 mg/Kg/die every 4 weeks). Immunophenotyping was performed at baseline, week 12 and week 24. A decline in B memory cells was observed during Tocilizumab therapy. Pre-switch and post-switch memory B cells decreased. The number of IgG+ and IgA+ B cells declined and in parallel, serum levels of IgA and IgG were diminished. In conclusion, the Authors suggest that IL-6 inhibition affects the B cell hyperactivity in RA patients.

## RESEARCH HIGHLIGHT

# Th17 cells, pathogenic or not? TGF- $\beta$ 3 imposes the embargo

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*Cellular & Molecular Immunology* (2013) 10, 101–102; doi:10.1038/cmi.2012.72; published online 11 February 2013

Th17 cells that express lineage-specific transcription factor RORC in human (and ROR $\gamma$ t in mice) and produce cytokines IL-17A, IL-17F, IL-21, GM-CSF and IL-22, constitute distinct subset of CD4<sup>+</sup> T cells.<sup>1</sup> Various reports indicated that Th17 cells have a critical role in the pathogenesis of several inflammatory and autoimmune diseases including rheumatoid arthritis, multiple sclerosis and psoriasis.<sup>2</sup> However, all IL-17-producing Th17 cells are not pathogenic. For example, existence of regulatory Th17 cells with immune-suppressive phenotype had been detected in small intestine.<sup>3</sup> As such, the factor(s) that govern(s) programming of non-pathogenic versus pathogenic Th17 cells is not completely known. Combination of TGF- $\beta$ 1 and IL-6 is sufficient to induce Th17 differentiation from naïve CD4<sup>+</sup> T cells, but IL-23 produced by the activated innate cells is critical for stabilizing Th17 cells and for acquiring pathogenic functions.<sup>1,4</sup> Also results from experimental autoimmune encephalomyelitis (EAE) in T-

bet-deficient mice demonstrated that encephalitogenic Th17 cells express Th1-transcription factor T-bet and encephalitogenicity of both Th1 and Th17 cells is governed by T-bet and not by T-cell lineage-specific cytokines IFN- $\gamma$  and IL-17.<sup>5</sup> Therefore, in a recent article in *Nature Immunology*,<sup>6</sup> Vijay Kuchroo and his colleagues aimed at identifying the IL-23-dependent effector molecule that imparts pathogenic phenotype to Th17 cells and the role of T-bet in the process.

By using EAE model, the authors first reconfirmed the role of IL-23 in the induction of pathogenic Th17 cells. Adoptive transfer of TGF- $\beta$ 1, IL-6 and IL-23-differentiated MOG-specific CD4<sup>+</sup> T cells led to development of severe disease in mice, whereas mild or no disease was observed when TGF- $\beta$ 1 and IL-6-differentiated T cells were transferred. Analysis of molecular changes in IL-23-exposed Th17 cell revealed considerable induction of TGF- $\beta$ 3 and provided the clue for the role of TGF- $\beta$ 3 in the induction of pathogenic Th17 cells.

Further, Th17-polarizing experiments in IL-23R-deficient cells revealed that IL-23 might not be essential for the initial induction of TGF- $\beta$ 3 and it could be induced by IL-6. Nevertheless, IL-23/IL-23R certainly required for further enhancement and maintenance of TGF- $\beta$ 3 expression. Additionally, the *in vivo* expression and induction of TGF- $\beta$ 3 in response to IL-23 was also confirmed by immunizing TGF- $\beta$ 3-eYFP fate-reporter mice with either MOG or MOG+IL-23. TGF- $\beta$ 3 was found to be endogenously expressed by CD4<sup>+</sup>, CD8<sup>+</sup>,  $\gamma\delta$ <sup>+</sup> T cells

and B cells, but not by myeloid cells. However, exposure to IL-23 mostly restricted TGF- $\beta$ 3 expression to Th17 cells.

Considering the association of IFN- $\gamma$ -secreting Th1 cells in the pathogenesis of autoimmune diseases, the authors intended to confirm if TGF- $\beta$ 3 could also be expressed by these cells. Deletion of TGF- $\beta$ 3<sup>+</sup> cells *in vivo*, in MOG-immunized mice led to selective ablation IL-17<sup>+</sup> T cells with no apparent changes in IFN- $\gamma$ <sup>+</sup> T cells. These results collectively implicated the nexus between TGF- $\beta$ 3 and pathogenic Th17 cells. Additional experiments also indicated that similar to TGF- $\beta$ 3, pathogenic Th17 cells could also be generated by a combination of IL-1 $\beta$ , IL-6 and IL-23, but both pathways were dependent on IL-23–IL-23R axis.

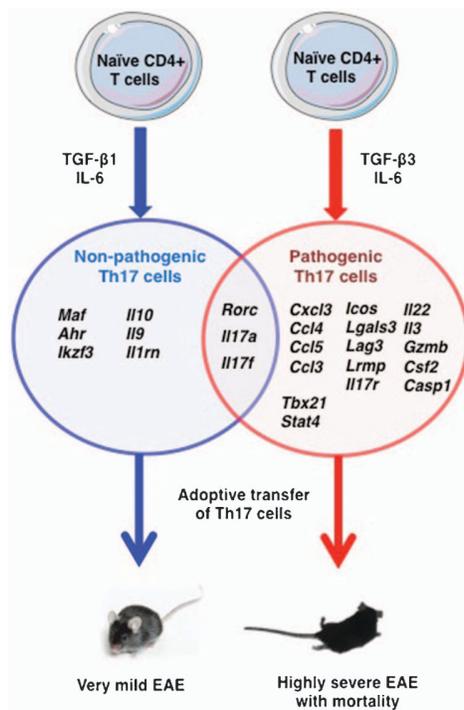
To determine if TGF- $\beta$ 3 has the potential to induce polarization of Th17 cells from naïve CD4<sup>+</sup> T cells, Lee *et al.* compared the expression of IL-17 and other Th17-associated molecules in the cells polarized in the presence of IL-6 along with either TGF- $\beta$ 3 or TGF- $\beta$ 1. Th17 cells polarized by TGF- $\beta$ 3 expressed *Rorc*, *Il17a* and *Il17f* similar to the cells polarized by TGF- $\beta$ 1. However, in contrast to TGF- $\beta$ 1-induced cells, TGF- $\beta$ 3-induced Th17 cells showed significantly higher expression of *Il22* and *Il23r* than the former. Furthermore, adoptive transfer of TGF- $\beta$ 3-induced but not TGF- $\beta$ 1-induced Th17 cells induced severe EAE in naïve mice with high mortality.

Of note, the authors found that both TGF- $\beta$ 3 and TGF- $\beta$ 1 signal through the same receptor (TGF- $\beta$ RII) for Th17

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**Figure 1** The molecular signatures of TGF- $\beta$ 1 and IL-6-induced non-pathogenic and TGF- $\beta$ 3 and IL-6-induced pathogenic Th17 cells. EAE, experimental autoimmune encephalomyelitis.

differentiation, suggesting that TGF- $\beta$ 3 and TGF- $\beta$ 1 deliver differential downstream signaling *via* TGF- $\beta$ RII. In fact, TGF- $\beta$ 3 and IL-6-differentiated Th17 cells had higher expression and phosphorylation of Smad1 and Smad5, and lower expression and phosphorylation of Smad2 and Smad3, which was in contrast to TGF- $\beta$ 1-induced Th17 cells.

Finally, to discover the molecular signature that distinguishes potentially pathogenic and non-pathogenic Th17 cells, Lee *et al.* differentiated CD4<sup>+</sup> naive T cells into Th17 cells under six different polarizing conditions. mRNA expression profiling of these cells by whole-genome microarray and subsequent principal component analysis categorized Th17 populations into three groups based on the differences in their molecular expression profiles.

Group I, consisted of Th17 cells induced by TGF- $\beta$ 1 and IL-6 with or without IL-23 and were mild or not pathogenic *in vivo*; group II included highly pathogenic cells induced by TGF- $\beta$ 3 and IL-6 or by IL-1 $\beta$ , IL-6; and group III included TGF- $\beta$ 3, IL-6 and IL-23- or IL-1 $\beta$ , IL-6 and IL-23-induced Th17 cells and had high expression of IL-23R similar to group II. In conclusion, the

authors demonstrated that Th17 cells induced by TGF- $\beta$ 3, IL-6 or IL-1 $\beta$ , IL-6 have similar molecular profile.<sup>6</sup> These pathogenic Th17 cells were marked by enhanced expression of genes encoding for specific cytokines, chemokines and transcription factors and downregulated expression of immunoregulatory genes (Figure 1).

The microarray analysis also identified increased expression of *Tbx21* (that encodes T-bet) in TGF- $\beta$ 3, IL-6-polarized pathogenic Th17 cells. The authors found that T-bet indeed has an important role for the induction of pathogenic signatures in Th17 cells. As TGF- $\beta$ 3 was also linked with pathogenic Th17 cells, the authors further investigated the possible link between T-bet and TGF- $\beta$ 3. They observed that T-bet-deficient Th17 cells did not induce EAE and had lower TGF- $\beta$ 3 expression. However, upon providing TGF- $\beta$ 3 exogenously, these cells overcame the necessity of T-bet, gained the pathogenicity and induced EAE upon adoptive transfer. These results indicated that T-bet is an integral part of pathogenic programming of Th17 cells and might regulate endogenous production of TGF- $\beta$ 3.

In conclusion, this study identified TGF- $\beta$ 3 as a 'driving force' to induce pathogenic Th17 cells. However, certain key issues remain to be answered: Is there any role for TGF- $\beta$ 3 in IL-1 $\beta$ , IL-6-induced pathogenic Th17 cells? As Th17 cells are important for the clearance of fungal infections and extracellular pathogens, do these protective Th17 cells function independent of TGF- $\beta$ 3? Does TGF- $\beta$ 3 play a critical role in driving potentially pathogenic Th17/Th2 phenotype in allergy and asthma?<sup>7</sup> Answering these questions and confirming the results in humans should pave the way to target TGF- $\beta$ 3 in several auto-inflammatory diseases.

## ACKNOWLEDGEMENTS

This work is supported by Institut National de la Santé et de la Recherche Médicale (INSERM), Université Pierre et Marie Curie, Université Paris Descartes and European Community's Seventh Framework Programme (FP7/2007-2013) under Grant Agreement No: 260338 ALLFUN and ANR-10-BLAN-1309 HYDROPHOBIN.

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